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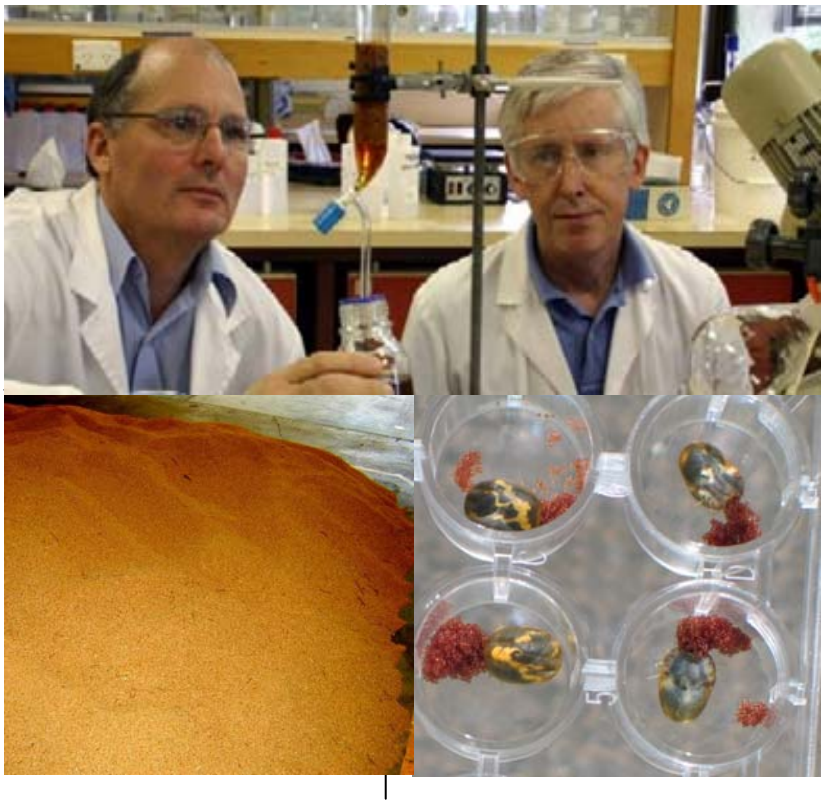
Final Report

Commercial products from bio-active extractives in cypress milling residues

FWPA Project PN04.2006



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The Department of Primary Industries and Fisheries (DPI&F) seeks to maximise the economic potential of Queensland's primary industries on a sustainable basis.

This report has been prepared by staff of Horticulture & Forestry Science and Animal Science Units within the Delivery Business Group, DPI&F.

Final Report

Project PN04.2006 – Commercial products from bio-active extractives in cypress milling residues

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Summary

Extractive components obtained from milling residues of white cypress were studied for chemical identity and bioactivity with a view to developing a commercial use for these components, thus increasing the value of the residues and improving the economics of cypress sawn wood production. Extracts obtained by solvent or steam extraction techniques from cypress sawdust were each fractionated by a range of techniques into groups of similar compounds. Crude extracts and fractions were screened against a range of agricultural pests and diseases, including two fungi, subterranean termites, fruit spotting bugs, two-spotted mites, thrips, heliothis, banana scab moths, silverleaf whiteflies, cattle tick adults and larvae, and ruminant gastrointestinal nematodes. Additional screening was undertaken where encouraging results were achieved, for two-spotted mites, thrips, silverleaf whiteflies, cattle tick adults and ruminant gastrointestinal nematodes. After considering degrees of efficacy against, and economic importance of, the agricultural pests, and likely production costs of extracts and fractions, the crude extract (oil) produced by steam distillation was chosen for further study against silverleaf whitefly. A useful degree of control was achievable when this oil was applied to tomato or eggplant at 0.1%, with much less harmful effects on a beneficial insect. Activity of the oil against silverleaf whitefly was undiminished 3.5 years after it was generated. There was little benefit from supplementing the extract with co-formulated paraffinic oil. From the steam distilled oil, fifty-five compounds were characterised, thirty-five compounds representing 92.478 % of the oil, with guaiol (20.8%) and citronellic acid (15.9%) most abundant. These two compounds, and a group of oxygenated compounds containing bulnesol and a range of eudesmols, were found to account for most of the activity against silverleaf whitefly. This application was recommended for first progression to commercialisation.

Introduction

Members of the genus *Callitris*, family Cupressaceae, are commonly called ‘cypress pines’ because of their resemblance to northern hemisphere cypresses (National Forest Library, 1998); however, they are not related and they are not true pines. Like all conifers, *Callitris* trees produce their seeds in cones. According to one estimate, 2.3 million hectares of *Callitris* is found in Australia and it is a part of many Australian ecosystems, from the arid tropics to the Australian Alps (Thompson and Johnson, 1986).

The most common species is white cypress, *Callitris glaucophylla* Joy Thomps and LAS Johnson. It is currently the most economically important *Callitris* species and is harvested from native forests. Two other important species are Bribie Island pine *Callitris columellaris* F. Muell. and *Callitris intratropica*, but these provide little, if any, timber. The three species have recently been combined as *Callitris columellaris* F. Muell. (Council of Heads of Australasian Herbaria, 2006)

White cypress grows as a tree to 30 m in height, with a single trunk, bark brown, rough and furrowed. The species is found in all mainland states of Australia, mostly on sandy soils, from isolated individuals to extensive forests, especially in inland districts. Extensive forests are found in the Tambo-Dalby-Inglewood region of Southern Queensland and the Baradine-Narrabri and Cobar districts of New South Wales (NSW).

White cypress wood is fairly hard and dense (675 kg/m^3) and is known as the world’s hardest coniferous timber. The strength and durability of the timber are important assets. It can resist decay and termites in ground contact for up to 50 years, and so has been widely used for ground contact applications, such as house stumps, fence posts and telephone poles, as well as for framing, flooring and furniture (National Forest Library, 1998).

Extractive compounds naturally present in the heartwood of durable timber species have long been known to confer durability to the wood because of their activity against the fungi and insects that would otherwise utilise the wood as food sources. This has been amply demonstrated with regard to white cypress (Dadswell and Dadswell (1931), Rudman (1965), French *et al*. (1979), Yazaki and Hillis (1997)). Examples have been reported of wood durability being reduced after removing these extractives and of naturally low-durability wood being enhanced by impregnation with solutions of them (Jiang *et al*. (2000), Kennedy and Powell (2000), Powell *et al*. (2000)).

Chemical compounds present in the suite of wood durability-conferring extractives are characteristic for a timber species, and the suite present in any species typically consists of many different compounds (Hillis, 1987). This chemical diversity is thought to broaden the spectrum of wood destroying organisms controlled, but it is also possible that multiple compounds form sub-sets active against individual organisms. Where such a sub-set of compounds consists of individual compounds quite diverse in their chemistry and mode of action, they may act synergistically.

In recent decades, many synthetic insecticidal compounds with useful activity profiles and acceptable environmental attributes have been developed and commercialised for use in agricultural and veterinary applications. But these often lose their usefulness in a relatively short period, through the gradual development by target pest populations of resistance to them (National Research Council, 1986). Perhaps it might be more difficult for an organism to develop resistance to a more chemically complex set of insecticidal compounds, such as might be extracted from insect-resistant durable wood?

Extractive compounds may be removed from the wood substrate in several ways, similar to those long used to obtain essential oils from a wide variety of plant materials. Steam-volatile extractive components may be removed by steam distillation, where finely divided wood in contact with boiling water and/or steam loses volatiles into the steam-laden atmosphere inside a retort. Steam and vapour exiting from the retort are condensed, whereupon the volatile essential oil spontaneously separates from the condensate water, forming a layer that can be drained off. The water, which contains a small amount of some partially soluble extractive compounds, may be re-used to extract another batch of wood. Alternatively, both volatile and non-volatile components may be removed by solvent extraction of finely-divided wood using one of many suitable organic solvents or solvent mixtures, under a range of conditions of temperature and pressure. After extraction, the solvent may be recovered for re-use.

About 200,000 cubic metres of white cypress logs are currently harvested annually from natural stands in inland parts of Queensland and NSW, and milled into sawn timber (DPI Forestry (2005), Forests NSW (2007)). From these data we estimate that over 20,000 tonnes/annum of sawdust or other milling residue is available for extraction, potentially yielding up to 2,000 tonnes of extractive compounds. The estimate has been reduced since this project was conceived, following the reservation of significant areas of the previously available cypress resource to National Park, and the closure of a number of cypress sawmills, particularly in New South Wales.

Successful development of a new commercial application for an extractive compound was expected to significantly improve the commercial value of the cypress milling industry, through the development of a cypress pine extractives/ waste reuse industry. The project offered the prospect of turning a disposal problem into profit generation.

Objectives and Deliverables

Key objectives of this project have been met -

- To explore the applicability of known insecticidal and fungicidal bioactivity of cypress heartwood extractives to the control of a wide range of wood and non-wood insect and fungal pests and diseases
- To discover and develop at least one high value agricultural pest and disease control application
- To evaluate and rank the cost-effectiveness of alternative industrial extraction technologies

Expected deliverables are ready for commercialisation -

- At least one agricultural pest or disease control application exploiting the natural insecticidal and/or fungicidal activity present in cypress sawdust
- Identification of the best process for industrial extraction of bioactivity from cypress sawdust, providing the industry with information to underpin an extraction infrastructure investment decision

Materials and Methods

Extraction

A batch of several cubic metres of fresh cypress sawdust was produced during routine sawing operations on white cypress at the mill of Logan Cypress, Narrabri, NSW, in November 2001. After mixing to uniformity, large sub-samples were immediately airfreighted to Brisbane and Sydney. The remainder was loaded into the industrial cypress oil distillation plant at Logan Cypress and subjected to steam distillation in accordance with their usual production process, yielding approximately 1.7% (dry weight basis) of recovered pale greenish-yellow oil.

In December 2001, 22 kg of the sawdust received in Brisbane was subject to atmospheric pressure extraction using methanol under reflux, by Solvent Services Ltd of Hemmant. After reaching boiling point and refluxing for four hours, the still was reconfigured to remove excess solvent by distillation, yielding approximately 18 L of a mobile light brown solution of cypress extractives in methanol/water. Remaining methanol and water were removed by rotary vacuum evaporation in the laboratory, yielding 1.27 kg of thick black extract similar in consistency to petroleum pitch. Yield was approximately 7.2% (solvent-free weight basis). This product (extract 'A') was stored in the laboratory at 22°C in the absence of light.

Also in December 2001, 47 kg of sawdust received in Sydney was subject to extraction using a confidential proprietary technique by Solvents Australia Ltd of Mona Vale. The process involved extraction under pressure using compressed (liquefied) R22 refrigerant gas, yielding 1.08 kg (approximately 2.7%, dry weight basis) of viscous brown oil. This oil (extract 'B') was immediately transferred to closed glass bottles and freighted to the DPI&F laboratory in Brisbane. On storage in the laboratory at 22°C in the absence of light, no components crystallised from the oil.

In April 2004, another batch of fresh sawdust produced during routine sawmilling operations was subjected to distillation at Logan Cypress, using the same plant and process as in November 2001. The resultant oil (extract 'C') was immediately placed in closed aluminium alloy Winchesters and freighted to the DPI&F laboratory in Brisbane. On storage in the laboratory at 22°C in the absence of light, guaiol (identified by mass spectrometry) gradually crystallised from the oil. Before subsequent sub-sampling of the crude extract, the container was warmed at 60°C until all guaiol re-dissolved.

Fractionation

First fractionation: After extensive preliminary experimentation on a range of adsorbent supports and many combinations of eluting solvents, crude extracts 'A', 'B', and 'C' minus guaiol were first subjected to open column chromatography as follows. Florisil™ (Magnesium silicate, 60-80 mesh) was heated to 140°C overnight and cooled, before placing 100 g in the base of a large (7 cm diameter) open glass column. Crude extract (100 g) was mixed with a further 1100 g portion of Florisil and added to the column. Fractions A1, A2, A3, A4 from crude extract 'A', B1 to B4 from crude extract 'B', and C1 to C4 from crude extract 'C' were successively eluted with *n*-hexane, toluene, ethyl acetate and methanol respectively. After removing the eluting solvent, each fraction was weighed. Overall recoveries from the column ranged from 88% to 101%. The fractions were used in the first series of screening trials against agricultural pests.

Second fractionation: Crude extracts 'A', 'B' and 'C' were also subjected to a different fractionation process using an automated preparative-scale reversed-phase high performance liquid chromatography (Prep HPLC) system, in order to generate a second set of fractions in which extractive components were grouped differently from the initial set. Crude extract (40 g) was dissolved in a minimum amount of methanol and diluted to a total volume of 100 mL prior to being placed in autoinjector vials. The Prep HPLC system was comprised of:

- Waters 600EF preparative scale pump system delivering a binary (methanol:water) solvent mixture at 10.0 mL/minute. The composition of the solvent mixture varied through the chromatographic run after each injection (Figure 1).
- Waters W717 Autoinjector fitted with a 2 mL sampling loop, loaded with 3 mL vials of methanol extract solution, from which a 1 mL injection was made for each of multiple runs.
- Waters preparative-scale C_{18} column, Part # 088500, µBondapak C_{18}, 15 cm length, 19 mm diameter.
- Waters 996 Photodiode Array Detector (PDA) operated in the MaxPlot mode (in which the full wavelength range of the diode array contributed to the output signal monitored.
- Waters Millenium32 instrument control, signal capture and integration software (v4.0) running on a PC interfaced to the system components.
- Waters Fraction Collector II collecting five fractions (labelled 11 to 15, Figure 1) from each run, receiving a start signal from the Auto injector and programmed to switch the detector effluent between 2.5 litre dark glass collecting vessels at run times also shown in Figure 1.

Prep HPLC chromatograms for crude extracts 'A', 'B' and 'C' are shown in Figure 2. Fractions A11 to A15, B11 to B15 and C11 to C15 were used in the second series of screening trials.

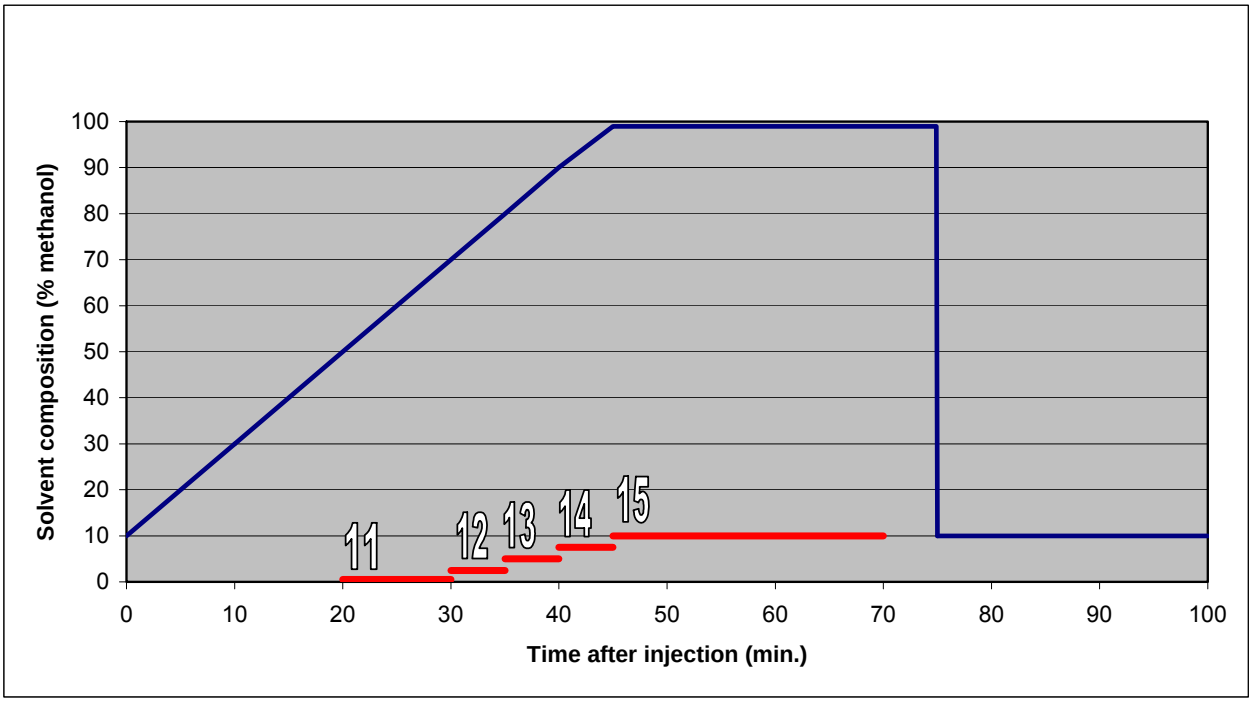

Figure 1: Solvent gradient and fraction collection timing for Prep HPLC fractionation: Blue line represents the varying solvent composition during the chromatographic run after each replicated injection. Red lines span the collection period for each fraction (labelled 11-15) collected.

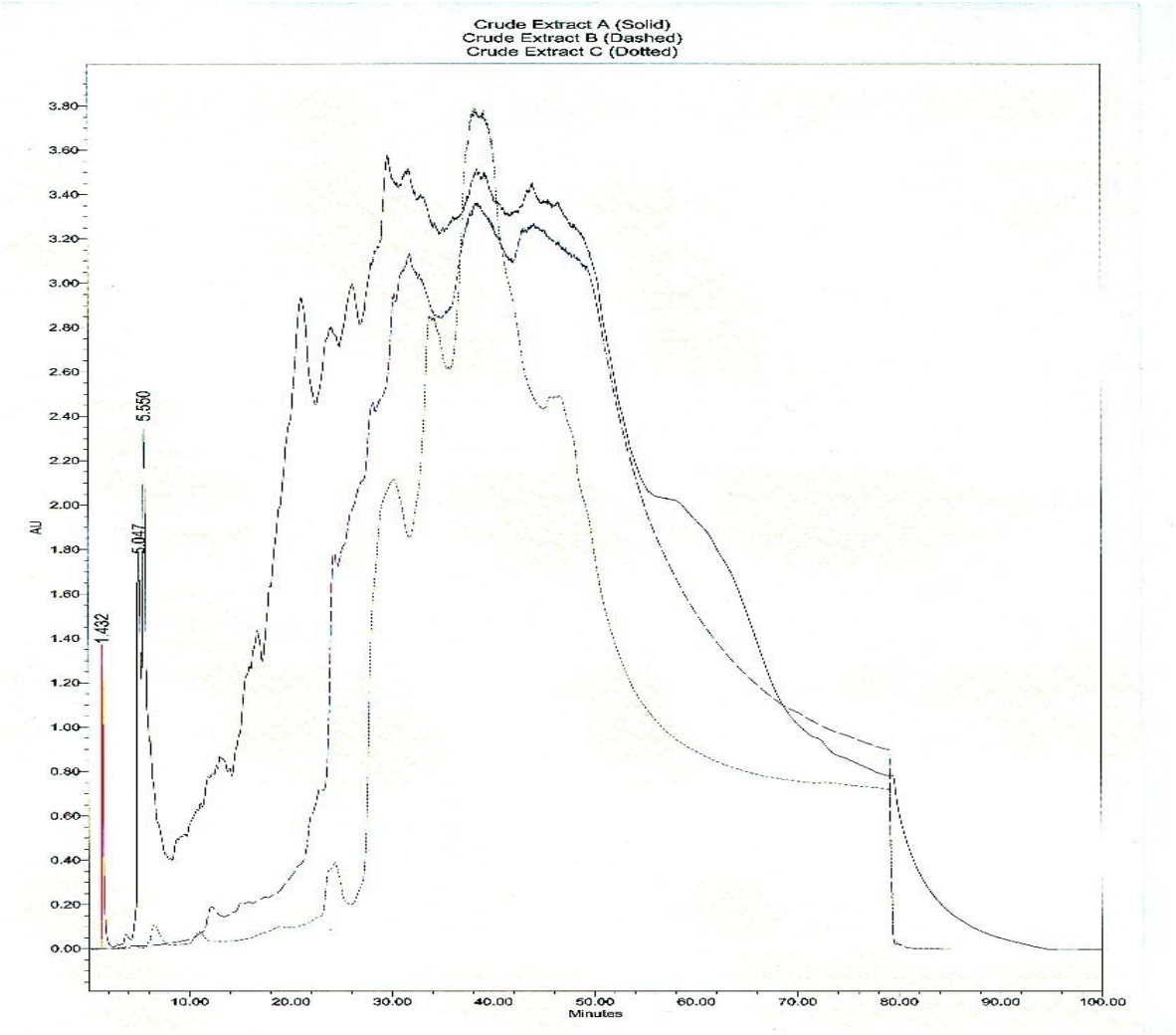

Figure 2: Preparative HPLC chromatograms for crude extracts 'A', 'B' and 'C'.

Third fractionation: A 50 g sub-sample of crude extract 'C' was fractionated by column chromatography (5 x 140 cm), on silica gel 60 (0.063–0.200 mm, Merck, 600 g) eluting with n-hexane (4L) followed by a gradient of *n*-hexane - ethyl acetate (1:0 to 0:1) and ethyl acetate - methanol (1:0 to 0.9:0.1) (2 L to 6 L of solvent mixture). Fifty-five column fractions (500 mL each) were collected and distilled under reduced pressure at 28–30°C. All fractions were monitored by thin layer chromatography (TLC) as described next below. Column fractions with similar TLC profiles were combined to yield eight different pooled fractions (C21 to C28). These fractions were kept in the dark at < 4°C. Similarly, a second 50 g sub-sample of the crude extract was fractionated in order to produce sufficient of each fraction for further screening. Fractions from both the columns were checked for similarity by TLC before combining (Table 1).

Table 1 Yields of each fraction from crude extract 'C' in the third fractionation experiment

First column		Second column		Total (%)(I+II)	Pooled fraction name
Fraction No.	Yield (%)	Fraction No.	Yield (%)		
1-8	10.1	1-7	11.9	11.0	C21
9-12	3.7	8-15	3.9	3.8	C22
13-17	2.0	16-17	4.2	3.1	C23
18-20	21.5	18-20	16.1	18.8	C24
21-32	43.8	21-34	44.7	44.3	C25
33-38	6.6	35-39	6.0	6.3	C26
39-44	4.6	40-47	7.0	5.8	C27
45-55	5.8	48-55	4.4	5.1	C28

Thin layer chromatography (TLC) of fractions: TLC was performed on pre-coated silica gel 60 F_{254} Merck, aluminium plates and detection of constituents was achieved by spraying with anisaldehyde sulphuric acid reagent containing 0.5 mL anisaldehyde, 90 mL ethanol and 10 mL sulphuric acid. All fifty-five column fractions were monitored on TLC by spraying with anisaldehyde-sulphuric acid reagent and heating the plates in an oven at 120°C for 30 to 120 seconds. Solvents used for running the TLC plates were *n*-hexane or a mixture of *n*-hexane and ethyl acetate.

Chemical analysis of extract and fractions: Gas chromatographic analysis with mass spectrometric detection (GC-MS) was carried out with an Agilent 5975 Inert Mass Selective Detector interfaced to an Agilent/HP 6890 GC system fitted with a capillary column of HP-5MS (5% phenyl methyl siloxane, length 30 m, inner diameter 250 µm, film thickness 0.25 µm). Injector, GC-MS interface, ion source and MS Quadrupole temperatures were maintained at 240, 280, 280, and 150°C respectively. The mass spectrometer was operated in electron-impact mode (EI) at 70 eV, in the scan range m/z 30–600. The oven temperature for volatile oil was programmed as follows: 50°C (held 1 min), 50 to 250°C (ramped at 3°C/min), 250°C (held 15 min). Helium flow rate through the column was 1 ml/min with a 30:1 split ratio. Crude extract or fraction was diluted to 0.5 or 1.0% (w/v) in diethyl ether and 0.2 or 1.0 µL was injected. A mixture of C_8-C_{20} *n*-alkanes was also separated under the above conditions and retention times were determined.

Kovats retention index for each component was determined relative to retention times of the series of n-alkanes. Identification of extractive components was based on comparison of the retention times, Kovats Indices and mass spectra with those of NIST Mass Spectral Library (version 2.0.d, build date Dec 2, 2005) and those described by Brecknell (1978), Brecknell and Carman (1978, 1979), Oyedeji *et al.* (1998), Doimo *et al.* (1999), Doimo (2001), Wilkinson and Cavanagh (2005), Watanabe *et al.* (2005), Beauchamp *et al.* (2006) and Adams (2007).

Refractionation of C25 Fraction: Pooled fraction C25 (25 g) was subjected to column chromatography (50 mm x 140 cm) on silica gel (500 g) eluting with a gradient of *n*-hexane – ethyl acetate (1:0 to 8:2). Fifty-five fractions (400 ml each) were collected and combined on the basis of their TLC profiles to yield seven sub-fractions A to G (Table 2). From sub-fractions C and D eluted with 8 % ethyl acetate - hexane mixture, one compound was spontaneously crystallized and identified as guaiol by GC-MS. The mother liquor from sub-fractions C (3.1 g) and D (12.8 g) was again separated by column chromatography on silica gel (150 g) and (180 g) respectively to obtain the richer fraction. The elution process was carried out with a mixture of ethyl acetate – *n*-hexane (1:19 to 1:4). Sub-sub-fraction C 6 (12.9%) and sub-sub-fraction C 9 -12 (33%) were obtained from sub-fraction C. Sub-sub-fraction D 6-13 (75.7%) was obtained from sub-fraction D. All the above fractions were analysed by GC-MS under the same running conditions as the earlier fractions.

Table 2 Yields of sub-fractions of fraction C25

Column fraction number	% of parent C25 fraction	Sub-fraction Name
1-16	1.2	A
17-19	2.4	B
20-25	13.28	C
26-33	52.0	D
34-40	18.4	E
41-46	2.72	F
47-55	4.48	G

Screening

First Screening: Specimens of 17 crude extracts or fractions from the first fractionation experiment were screened against two-spotted mite, fruit-spotting bug, thrips, banana scab moth, silverleaf whitefly, cattle tick larvae and adults, sheep gastrointestinal nematodes, heliothis larvae, subterranean termites, and fungal disease organisms *Rosellinia necatrix* and *Alternaria mali,* using methods described in the Appendices. Fraction specimens were coded during screening (Table 3):

Table 3 Fraction specimen codes for the first screening trial

	Methanol extract	Refrigerant gas extract	Steam-distilled oil
Crude Extract	'A'	'B'	'C'
Hexane fraction	A1	B1	C1
Toluene fraction	A2	B2	C2
Ethyl Acetate fraction	A3	B3	C3
Methanol fraction	A4	B4	C4
From original extract C:			
guaiol			C5
oil minus guaiol			C6

Second Screening: Specimens of 19 crude extracts or fractions from the second fractionation experiment were screened against pests where positive outcomes were observed in the first set of screening experiments, *viz*, two-spotted mite, thrips, silverleaf whitefly, cattle tick adults and sheep gastrointestinal nematodes, using methods described in the Appendices. Specimens were again coded (Table 4):

Table 4 Fraction specimen codes for the second screening trial

	Methanol extract	Refrigerant gas extract	Steam-distilled oil
Crude Extract	'A'	'B'	'C'
PrepHPLC 20–30 minute fraction	A11	B11	absent
PrepHPLC 30–35 minute fraction	A12	B12	C12
PrepHPLC 35–40 minute fraction	A13	B13	C13A & C13B
PrepHPLC 40–45 minute fraction	A14	B14	C14
PrepHPLC 45–70 minute fraction	A15	B15	C15
From original extract C:			
guaiol			C5
oil minus guaiol			C6

Third Screening: Specimens of crude extract 'C' and fractions from the third fractionation experiment were supplied blind for screening against silverleaf whitefly using methods described in Appendix L. Fraction specimens were coded C21 to C28 (Table 1), together with C29 (guaiol) and C30 (crude extract 'C'). Sub-fractions from the refractionation of fraction C25 were supplied blind as C31 to C37 (Table 5).

Table 5 Codes for subfractions from Fraction C25 of crude extract 'C' for the third screening trials

Code	**Indentity**
C31	Refraction D of C25
C32	Refraction E of C25
C33	Refraction G of C25
C34	3:1 mixture of Refraction G and guaiol
C35	1:1 mixture of Refraction G and guaiol
C36	1:3 mixture of Refraction G and guaiol
C37	guaiol

Additional experiments on silverleaf whitefly were conducted using crude extract 'C' and mixtures of crude extract 'C' with a commercially-available hydrocarbon oil based product (Appendix M). Activity against a beneficial insect was also evaluated (Appendix N).

Results and Discussion

Screening trials

First screening experiments: Although it was intended to screen against mosquito larvae, agreement could not be reached with James Cook University on IP issues, and the R&D Steering Committee concurred with the omission of this aspect of the work. The committee also agreed to suspend screening against subterranean termites when several screening techniques were incompatible with the relatively volatile compounds present and a patent application for a related termite control application was discovered. Extracts and fractions were quite ineffective against fruit spotting bug adults (Appendix A). As outcomes of screening against subterranean termites, cattle tick larvae and *Heliothis* larvae were inconclusive, data have not been provided for these aspects.

Detailed first screening effectiveness data are provided in Appendices cited for those organisms for which more conclusive data were obtained, *viz* two-spotted mite, thrips, banana scab moth, silverleaf whitefly, adult cattle tick and ruminant gastrointestinal nematode. In summary:

- *Two-spotted mite* (Appendix A) Fractions A2, A4 and C4 showed activity against this pest, although less than the abamectin standard. However, because resistance develops rapidly, new miticides are continually needed, so this organism was selected for further work.
- *Thrips* (Appendix C). The 'C' extract and all of its fractions except C5 (guaiol) provided control of thrips equivalent to the standard dimethoate, the only insecticide registered for thrips on lupins, although it's continued registration is uncertain. It was selected for further work.
- *Banana scab moth larvae* (Appendix E). A1, B, B4 and C5 showed some repellence, but extracts and fractions did not produce mortality. It was not included in further screening.
- *Silverleaf whitefly* (Appendix F). Rapid knockdown was indicated from B4 and C6, but significant mortality was observed from A2, A4, B4, C5 and C6. B4 and C5 markedly reduced egg laying, egg hatchability and nymph establishment. The activity of C5 here is interesting, when compared with its apparent ineffectiveness against thrips. This raised the possibility that an easily-achieved guaiol removal from the steam volatile oil could form the basis of products for both whitefly (the guaiol) and thrips (the remainder). Whitefly is an increasingly serious problem across many crops, and rapidly develops resistance to synthetic insecticides. It was selected for further work.
- *Cattle tick* (Appendix H). Crude 'C' extract and the hexane fractions A1, B1 and C1 provided useful mortality of adult cattle tick in the laboratory. It was selected for further work.
- *Ruminant gastrointestinal nematodes* (Appendix J). Fractions C2, C4 and C6 from the steam volatile oil were particularly toxic to the test organism from sheep. It was selected for further work.
- *Rosellinia necatrix* and *Alternaria mali* (Appendix O). Extracts and fractions provided little control of these fungal organisms, so no further work was undertaken on fungi.

Second screening experiments: Detailed second screening results are provided in the appendices, for two-spotted mite (Appendix B), thrips (D), silverleaf whitefly (G), adult cattle tick (I) and ruminant gastrointestinal nematode (K). Relative degrees of activity are summarised in Table 6.

Outcomes of the second screening trial generally corresponded with and confirmed the outcomes of the initial trial. Potentially useful activity was observed against each of the pests for which screening was conducted. The project plan called for one or more potential pest control applications to be developed. It was never envisaged that project resources would be sufficient to develop applications for all positive observations of bioactivity, so it was necessary to choose between them.

Table 6. Combinations of extract (or fraction) and pest screened in the second set of trials (grey shading).

Pest:	Two-spotted mite	Thrips	Silver-leaf whitefly		Cattle tick	Ruminant gastrointestinal nematode
	adult	adult	nymph	adult	adult	larva
Appendix	**B**	**D**	**G**	**G**	**I**	**K**
Crude extract or fraction identifier						
Crude Extract 'A'	+++	++		+++		+
Fraction A11	++			+		
Fraction A12	+++			+++		
Fraction A13	+++		++	+++		
Fraction A14	+++		+	+++		
Fraction A15	++			+		
Crude Extract 'B'	+	++		+++		+
Fraction B11				+		
Fraction B12				+++	++	
Fraction B13			++	+++		
Fraction B14				+++		
Fraction B15				+++		
Crude Extract 'C' minus guaiol	+	++		++	++	+
Fraction C12	+	+		+++		+++
Fraction C13A	+	++		++		++
Fraction C13B		++		+	+++	+
Fraction C14	+			++	+++	+
Fraction C15		++				
Fraction C5 (guaiol)				++		

Note 1: Symbols indicate degree of activity against the pest observed:

+++ (high degree of activity, comparable to standard (positive) control treatment)*****;

++ (intermediate degree of activity);

+ (somewhat better activity than the blank (negative control);

Grey cells without a symbol signify negligible activities.

***Note 2**: no positive control was used in screening against cattle tick.

Choice of a target pest control application: It was recognised by the R&D Steering Committee that veterinary applications would be more time consuming and expensive to develop than plant crop pests, due to the multiple additional procedures required to test and register products for use on animals. Therefore a focus on a potential crop application was preferred and several opportunities had been identified and confirmed during the screening trials.

In choosing between the three cypress extraction procedures evaluated in this project, it was recognised that yields and costs of production would vary considerably:

- Steam-distilled volatile oil (extract 'C') could be produced using the simplest technology, almost certainly with the lowest capital and operating costs. It requires a steam pressure vessel which can probably be heated by burning extracted sawdust. Steam and volatile cypress compounds emerging from the extraction vessel are condensed, and the oil floats on

top of the water. Water is recovered and recycled. No solvents are used. However, it also produces the lowest yield (about 1.7% of the dry weight of the sawdust extracted).

- Alcoholic solvent extraction (extract 'A') requires more complex and more expensive technology. No elevated pressures are necessarily involved, but the solvents are flammable and dangerous. After boiling the sawdust in the solvent for an hour or more, extractive-laden solvent is removed from the vessel and concentrated by removal and recycling of most of the solvent. A vacuum pump and vacuum-rated vessel is required. Despite solvent recovery, an estimated 10–15% of the solvent would be lost for each charge as the extracted sawdust is effectively saturated with solvent. Operating costs would therefore be considerably higher than for steam volatile oil production. Yields are also much higher (about 7.2% dry weight basis)
- The third extraction technique involves compressed refrigerant gas. This becomes a liquid under pressure and is pumped through the charge of sawdust to remove the extractive components (extract 'B'). On completion and removal of the extractive-laden fluid, virtually all of the solvent is recovered by pressure reduction in a second closed vessel, the gas being pumped off and re-compressed to liquid for the next charge. Gases are extremely expensive, but net usage would be very low. Plant capital cost would be very high. Yields are intermediate (about 2.7% dry weight basis)

In choosing a target plant crop and pest, factors considered included:

- the amount of the crop grown, size of the market, and whether expanding or contracting,
- the effectiveness, cost and registration status of current control methods, and
- opportunities for substituting a cypress based product for the current control method, or for incorporating it into an existing or a novel or existing integrated pest management system

In developing a cypress-extractive based pest control application for a chosen plant crop and pest, factors considered included:

- delivery (application) methods
- product formulation questions (formulation cost, stability, effectiveness)
- phytotoxicity (damage to the plant caused by the application of the extract)
- taint or chemical residues in the fruit or vegetable from cypress extractive components and applicable withholding periods
- whether efficacy observed in the lab or glasshouse is maintained under field conditions,
- economics of the application when compared with currently registered treatment(s)
- additional data required for registration of the product

It was suggested that registration requirements for an 'organic' naturally bioactive extract may be less onerous than for more refined mixtures or individual components of the extract. In this case, the development of an application based upon a crude extract might be preferable to the use of any of the fractions, particularly when supported by the economic advantage of eliminating substantial fractionation costs. Choosing a crude extract in the target commercial application would not render past screening work on fraction activity superfluous. For production quality control (batch to batch product standardisation), it is essential to understand the relative contributions of extractive components to product efficacy.

The following opportunities, arising from the screening trials, were considered for further research:

- **Two-spotted mite**. Two spotted mite is a problem for a wide range of horticultural crops (Appendix A). Extract 'A' showed good activity against this pest. Extract 'A' at 0.2% (2000 mg/L) was not far less effective than the abamectin standard at 16.2 mg/L. Extracts 'B' and 'C' were much less effective. Because the pest rapidly develops resistance, new miticides are continually needed and development of resistance against the chemically very complex cypress extract may be much slower than for any single synthetic insecticide. However, it appears to be increasingly likely that mite control will be achieved through biological control using natural and introduced predators.
- **Thrips**. Thrips are a problem for a very wide range of horticultural crops, including stone and pome fruit, citrus, strawberry, tomato, onion, cut flowers. In the initial screening trial, the 'C' extract and all of its fractions except guaiol (C5) at 0.2% provided control of thrips equivalent to the dimethoate standard at 300 mg/L. In the second trial, all three crude extracts 'A', 'B' and 'C' at 0.2% provided control almost as effective as the fenthion standard at 390 mg/L. There is uncertainty about the continued registration of dimethoate, the only insecticide currently registered for thrips on lupins.
- **Silverleaf whitefly**. Whitefly is an increasingly serious problem across many crops, including cotton, soybean, tomato and French bean, with over 500 host plants, and has shown a remarkable ability to develop resistance to synthetic insecticides (University of California, 2003). In the first screening trial, rapid knockdown was indicated from B4 and C6, but significant adult mortality was observed from A2, A4, B4, C5 and C6, while B4 and C5 markedly reduced egg laying, egg hatchability and nymph establishment. In the second trial, all three crude extracts and many of the fractions at 0.2% were as effective against adult whitefly as the imidacloprid-containing standard at 50 mg/L a.i., but most of the extracts and fractions were not as effective against nymphs as the standard. The activity of guaiol against whitefly, suggested in the first screening, was confirmed in the second. As guaiol appears to be ineffective against thrips, a possible use consists of the separation of guaiol from crude extract 'C'. Crude extract 'C' could form the basis for products against both whitefly (the guaiol component) and thrips (the remainder of 'C').
- **Cattle tick adults**. Crude extract 'C' and a couple of its fractions provided useful mortality of adult ticks in the laboratory in both screening trials.
- **Ruminant gastrointestinal nematodes**. There was little difference between each of the crude extracts, but none of them was as effective as an alternative essential oil distilled from foliage of another plant species. As in the initial screening trial, a couple of fractions from crude extract 'C' were again particularly toxic to larvae of the test organism in the lab.

After wide consultation with pest and crop specialists in the Department of Primary Industries and Fisheries, and in consideration of the likely lower capital cost of extraction facilities for the volatile oil 'C', the R&D Steering Committee supported concentration upon use of extract 'C' against silverleaf whitefly for the remainder of the project. Additional research addressed efficacy issues likely to arise during an anticipated subsequent commercialisation process for a whitefly control product.

Third screening experiments: Additional experimental work was accordingly undertaken using crude extract 'C' and it's fractions, subfractions and components against silverleaf whitefly (Appendices L and M). Analysis of these data is included under the heading 'Relative contribution of components to activity', below.

Chemical composition

Quantitative analysis of crude extract 'C', and of each pooled fraction and sub-fraction produced in the third fractionation experiment produced the data given in Tables 7 to 15. In these Tables, RT represents retention time on the gas chromatographic column, KI represents the Kovats retention index, and identification methods are either KI or MS (Mass spectrometry) or both.

Table 7 Composition of crude extract 'C' by GC-MS.

Peak No.	Compounds	% found	RT (min)	KI	Identification
1.	α-Pinene	0.085	6.567	939	MS, KI
2.	6-methyl-5-heptene-2-one	1.762	8.682	1000	MS, KI
3.	Methyl citronellate	0.203	20.009	1274	MS, KI
4.	Methyl geranate	2.113	22.642	1336	MS, KI
5.	Citronellic acid	15.935	23.353	1353	MS, KI
6.	Neric acid	0.290	24.602	1382	MS, KI
7.	β-Elemene	0.568	25.171	1395	MS, KI
8.	Aromadendrene	0.223	26.214	1421	MS, KI
9.	α-Guainene	0.250	27.053	1443	MS, KI
10.	α-Humulene	0.311	27.603	1456	MS, KI
11.	Aromadendrene<allo>	< 0.05	28.256	1472	MS, KI
12.	(-)-Eudesma-1,4(15),11-triene	0.865	28.366	1475	MS, KI
13.	Guaiene<cis-β->	1.295	28.541	1479	MS, KI
14.	EE-methyldehydrogeranate	2.901	28.651	1482	KI
15.	β-Selinene	6.414	28.916	1488	MS, KI
16.	α-Selinene	5.387	29.291	1497	MS, KI
17.	Elemol	0.577	31.601	1559	MS, KI
18.	Guaiol	20.799	33.451	1606	MS, KI
19.	10-epi-γ-Eudesmol	0.268	34.557	1638	MS, KI
20.	γ-Eudesmol	3.644	34.654	1640	MS, KI
21.	β-Eudesmol	5.822	35.301	1658	MS, KI
22.	α-Eudesmol	5.127	35.417	1662	MS, KI
23.	7-epi-α-Eudesmol	1.461	35.605	1667	MS, KI
24.	Bulnesol	9.634	35.980	1677	MS, KI
25.	Methyl-cis-isocosticate	0.118	40.081	1794	MS,KI
26.	Elemanolide isomer 1	0.157	40.838	1817	MS
27.	Callitrin	0.656	41.446	1835	MS
28.	Eudesmanolide isomer	0.206	42.843	1878	MS
29.	Dihydrocolumellarin	1.994	44.053	1915	MS
30.	Germacranolide	0.307	44.324	1923	MS
31.	Elemanolide isomer 2	0.092	45.127	1950	MS
32.	Callitrisin	0.956	45.340	1956	MS
33.	Columellarin	1.268	45.592	1964	MS
34.	Elemanolide 3	0.278	45.773	1970	MS
35.	Dihydrocallitrisin	0.462	46.142	1981	MS

Table 8 Percentage Composition of Fraction No. C21

Peak	Compounds	% found	RT (min)	KI	Identification by
1	α-Pinene	0.227	6.528	938	MS, KI
2	o-Cymene	<0.200	9.724	1032	MS, KI
3	Limonene	0.271	9.827	1035	MS, KI
4	β-Elemene	2.461	25.080	1394	MS, KI
5	Aromadendrene	0.659	26.122	1421	MS, KI
6	α-Guainene	1.456	26.959	1442	MS, KI
7	Aromadendrene<allo->	0.800	28.172	1473	MS, KI
8	(-)-Eudesma-1,4(15),11-triene	3.280	28.269	1475	MS
9	β-Selinene	43.992	28.858	1489	MS, KI
10	Valencene	1.521	28.929	1491	MS, KI
11	α-Selinene	33.081	29.227	1498	MS, KI
12	α-Murrolene	0.504	29.466	1504	MS, KI
13	α -Bulnesene	0.738	29.660	1509	MS, KI
14	α -Amorphene	0.371	29.964	1518	MS, KI
15	δ-Cadinene	0.621	30.372	1529	MS, KI

Table 9 Percentage Composition of Fraction No. C22

Peak	Compounds	% found	RT (min)	KI	Identification by
1	o-Cymene	<0.100	9.717	1032	MS, KI
2	β-Elemene	5.445	25.074	1394	MS, KI
3	Aromadendrene	0.522	26.115	1421	MS, KI
4	α-Guainene	0.698	26.950	1442	MS, KI
5	α-Humulene	7.396	27.499	1456	MS, KI
6	(-)-Eudesma-1,4(15),11-triene	5.917	28.263	1475	MS
7	Guainene<cis-β->	7.818	28.450	1479	MS, KI
8	β-Selinene	35.949	28.845	1489	MS, KI
9	Valencene	1.210	28.929	1491	MS, KI
10	α-Selinene	28.155	29.214	1498	MS, KI
11	α-Murrolene	0.547	29.459	1504	MS
12	α -Bulnesene	0.629	29.654	1509	MS, KI
13	β-Bisabolene	0.922	29.802	1513	MS, KI
14	γ-Cadinene	0.390	29.958	1517	MS, KI
15	Selina-3,7(11)-diene	0.649	30.068	1520	MS
16	δ-Cadinene	0.789	30.365	1529	MS, KI
17	α-Calacorene	0.273	31.135	1549	MS, KI
18	Guaiazulene	0.271	36.051	1681	MS

Table 10 Percentage Composition of Fraction No. C23

Peak	Compounds	% found	RT (min)	KI	Identification by
1	6-Methyl-5-heptene-2-one	5.860	8.527	998	MS, KI
2	1,8-Cineole	0.244	10.125	1042	MS, KI
3	3-Pinanone	0.335	15.474	1171	MS
4	4,8-Dimethyl-7-nonen-2-one	0.514	18.450	1240	MS
5	Methyl citronellate	2.155	19.834	1272	MS, KI
6	Methyl geranate	27.267	22.519	1335	MS, KI
7	α-Humulene	0.262	27.493	1456	MS, KI
8	EE-methyldehydrogeranate	20.972	28.547	1482	KI
9	1-Acetyl-4,6,8-trimethylazulene	0.507	35.501	1666	MS

Table 11 Percentage Composition of Fraction No. C24

Peak	Compounds	% found	RT (min)	KI	Identification by
1	6-Methyl-5-heptene-2-one	5.383	8.566	998	MS, KI
2	Methyl citronellate	0.624	19.879	1273	MS, KI
3	Methyl geranate	7.931	22.506	1335	MS, KI
4	Citronellic acid	25.771	23.030	1347	MS, KI
5	Neric acid	0.791	24.388	1379	MS, KI
6	EE-methyldehydrogeranate	6.344	28.515	1482	KI
7	Guaiol	29.740	33.276	1604	MS, KI
8	10-epi-γ-Eudesmol	0.395	34.427	1637	MS, KI
9	γ-Eudesmol	0.986	34.512	1639	MS, KI
10	7-epi-α-Eudesmol	0.532	35.449	1665	MS, KI
11	Bulnesol	1.599	35.825	1675	MS, KI
12	Columellarin<dihydro->	7.154	43.898	1913	MS
13	Callitrisin	1.093	45.178	1955	MS
14	Columellarin	3.710	45.444	1963	MS

Table 12 Percentage Composition of Fraction No. C25

Peak	Compounds	% found	RT (min)	KI	Identification by
1	Citronellic acid	12.605	22.928	1345	MS, KI
2	Elemol	0.779	31.443	1557	MS, KI
3	Guaiol	32.842	33.287	1604	MS, KI
4	10-epi-γ-Eudesmol	0.587	34.421	1637	MS, KI
5	γ-Eudesmol	6.010	34.499	1638	MS, KI
6	β-Eudesmol	10.900	35.143	1657	MS, KI
7	α-Eudesmol	10.008	35.260	1660	MS, KI
8	7-epi-α-eudesmol	1.849	35.443	1665	MS, KI
9	Bulnesol	18.044	35.825	1675	MS, KI
10	Callitrin	0.945	41.277	1833	MS
11	Columellarin<dihydro->	1.525	43.879	1913	MS
12	Germacranolide	0.537	44.147	1922	MS
13	Callitrisin	0.901	45.182	1955	MS
14	Columellarin	0.222	45.425	1963	MS

Table 13 Percentage Composition of Fraction No. C26

Peak	Compounds	% found	RT (min)	KI	Identification by
1	6-Methyl-5-heptene-2-ol	0.651	8.915	1009	MS, KI
2	β-Citronellol	4.829	18.497	1241	MS, KI
3	Citronellic acid	41.217	23.127	1349	MS, KI
4	Elemol	0.843	31.444	1557	MS, KI
5	Guaiol	6.059	33.255	1603	MS, KI
6	γ-Eudesmol	1.711	34.493	1639	MS, KI
7	β-Eudesmol	5.570	35.132	1656	MS, KI
8	α-Eudesmol	6.777	35.250	1660	MS, KI
9	Bulnesol	5.322	35.801	1675	MS, KI
10	Columellarin	< 0.1	45.411	1962	MS

Table 14 Percentage composition of Fraction No. C27 and C28

Peak	Compounds	% found	RT (min)	KI	Identification by
		C27			
1	Citronellic acid	83.546	23.193	1351	MS, KI
2	Columellarin	0.627	45.405	1962	MS
		C28			
1	Citronellic acid	78.103	22.966	1346	MS, KI

Table 15 Composition of sub-sub-fractions and sub-fractions of Fraction C25.

Sub-fraction	Compounds	% found	RT (min)	RI	Identification by
C (6)	Columellarin<dihydro->	77.642	43.302	1916	MS
	Callitrisin	6.952	44.570	1957	MS
	Columellarin	10.302	44.822	1965	MS
C (9-12)	Citronellic acid	12.003	22.797	1355	MS, KI
	Guaiol	85.523	32.881	1610	MS,KI
D (6-13)	Citronellic acid	0.779	22.402	1346	MS,KI
	Elemol	0.496	30.960	1560	MS,KI
	Guaiol	36.459	32.804	1608	MS,KI
	10-epi-γ-Eudesmol	1.114	33.910	1640	MS,KI
	γ-Eudesmol	9.101	34.000	1642	MS,KI
	β-Eudesmol	9.762	34.634	1660	MS,KI
	α-Eudesmol	8.063	34.757	1663	MS,KI
	7-epi-α-eudesmol	2.414	34.938	1668	MS,KI
	Bulnesol	22.630	35.326	1679	MS,KI
	Callitrin	2.086	40.786	1838	MS
	Columellarin<dihydro->	0.464	43.360	1917	MS
	Germacranolide	1.041	43.645	1927	MS
	Callitrisin	1.004	44.680	1961	MS
E	Borneol	0.565	15.267	1178	MS,KI
	Citronellic acid	2.630	22.441	1347	MS,KI
	Elemol	2.037	30.941	1560	MS,KI
	Guaiol	2.124	32.745	1606	MS,KI
	γ-Eudesmol	7.459	33.981	1642	MS,KI
	β-Eudesmol	30.093	34.654	1660	MS,KI
	α-Eudesmol	23.919	34.770	1663	MS,KI
	7-epi-α-eudesmol	1.423	34.925	1668	MS,KI
	Bulnesol	25.022	35.320	1678	MS,KI
	Elemanolide isomer	0.424	40.152	1818	MS
F	6-Methyl-5-heptene-2-ol	0.268	8.663	1013	MS,KI
	α-Terpineol	1.601	16.380	1201	MS,KI
	Citronellic acid	22.977	22.713	1353	MS,KI
	Elemol	5.145	30.915	1559	MS,KI
	γ-Eudesmol	1.008	33.975	1641	MS,KI
	β-Eudesmol	21.501	34.615	1659	MS,KI
	α-Eudesmol	26.636	34.738	1664	MS,KI
	Bulnesol	5.418	35.275	1677	MS,KI
	Elemanolide isomer	4.276	40.146	1818	MS
	Elemanolide isomer	1.601	45.055	1973	MS
G	Citronellic acid	99.479	23.036	1359	MS,KI
	Neric acid	0.521	24.058	1384	MS,KI

Thirty five compounds comprised 92.5% of crude extract 'C', with guaiol (20.8%), citronellic acid (15.9%), bulnesol (9.6%), β-selinene (6.4%), β-eudesmol (5.8%), α-selinene (5.4%), α-eudesmol (5.1%), γ-eudesmol (3.6%) and EE-methyldehydrogeranate (2.9%) as the main constituents (contributing ≈3% or more). 6-Methyl-5-heptene-2-one, methylgeranate, guainene<cis-β>, 7-epi-α-eudesmol, dihydrocolumellarin, columellarin were also present in significant quantities (>1 %). The results are in agreement with those of other cypress oil studies (Doimo *et al*. (1999), Doimo (2001)), but aromadendrene, α-humulene, aromadendrene<allo>, guainene<cis-β>, are here reported for the first time from whole cypress oil.

Furthermore, ten γ-lactones are also present in the oil (Doimo *et al*. (1999), Doimo (2001)). Callitrin, dihydrocolumellarin, germacranolide, callitrisin, columellarin and dihydrocallitrisin collectively constituted 5.6% of the oil. Other γ-lactones having elemanolide and eudesmanolide structures constituted 0.73% of the oil. We also report here the germacranolide having the same fragmentation pattern as reported by Brecknell (1978) and Doimo *et al* (1999).

After fractionation of the oil on silica gel into eight major fractions, further analysis by GC-MS allowed an additional twenty compounds to be identified. These compounds have significant percentage in the fractions, but in the oil they are present only in traces. Except 6-methyl-5-hepten-2-ol, all twenty three compounds including aromadendrene, α-humulene, aromadendrene<allo>, guainene<cis-β> are reported here for the first time from cypress oil.

Stability of bioactivity

An estimate of the stability of the activity of cypress extracts was derived from data generated during repeated screening experiments against silverleaf whitefly over more than three years.

Crude extracts 'A' and 'B' generated in December 2001 were screened twice, in June 2005 (Appendix F) and in March 2006 (Appendix G), by the same staff using the same methodology. A 'blank' (negative control) treatment included on each occasion facilitated an allowance for 'between experiment' variation. We used mortality data from the longest contact time studied in each case, to ensure that the full effect of the treatment has been captured, and because counts made at the conclusion of the experiment were more accurate than those made after intermediate contact periods. Data summarised in Tables 15 and 16 do not indicate any loss of silverleaf whitefly activity on the part of either extract over the period from 42 months to 51 months – if anything, an increase is indicated, although such an increase would not be expected and is not claimed.

Table 15 Stability of crude extract 'A' activity against silverleaf whitefly (SLW) over two experiments

Age of extract at screening date (months)	Maximum contact period (days)	SLW mortality of 0.2% 'A' treatment after max. contact period 'M' (%)	SLW mortality of control treatment after same period 'C' (%)	Mortality adjusted for control variation ('M' – 'C') (%)
42	7	67.9	29.8	37.1
51	5	100	45.0	55.0

Table 16 Stability of crude extract 'B' activity against silverleaf whitefly (SLW) over two experiments

Age of extract at screening date (months)	Maximum contact period (days)	SLW mortality of 0.2% 'B' treatment after max. contact period 'M' (%)	SLW mortality of control treatment after same period 'C' (%)	Mortality adjusted for control variation ('M' – 'C') (%)
42	7	61.3	29.8	31.5
51	5	100	45.0	55.0

Crude extract 'C', generated in April 2004, was screened against silverleaf whitefly in June 2005, March 2006 and November 2007 (Appendices F, G and M respectively). As the latter experiment was conducted in a different laboratory on a different host plant by a different researcher, the data will not support a rigorous stability analysis, but the consistent inclusion of the control treatment again provided a basis for comparison. Table 17 summarises data from these experiments.

Table 17 Stability of crude extract 'C' activity against silverleaf whitefly (SLW) over three experiments

Age of extract at screening date (months)	Maximum contact period (days)	SLW mortality of 0.2% 'C' treatment after max. contact period 'M' (%)	SLW mortality of control treatment after same period 'C' (%)	Mortality adjusted for control variation ('M' – 'C') (%)
14	7	57.6	29.8	27.8
23	5	81.9	45.0	36.9
43	4	42.5	7.5	35.0

This analysis does not indicate loss of bioactivity of crude extract 'C' over the period from 14 months to 43 months from the date of distillation from fresh sawdust.

While it was not possible to rule out losses of activity of crude extracts 'A' and 'B' in the 42 months that elapsed between production of the extracts and evaluation of bioactivity, it appeared quite safe to consider the extract recommended for initial commercialisation (crude extract 'C') to retain its original stability for at least 3.5 years from the date of production.

Relative contribution of components to activity against silverleaf whitefly

A visual comparison of activity data from the third series of screening experiments against silverleaf whitefly (Experiments 1 & 2, Appendix L) with the analysed chemical composition (Tables 9 to 14) of the fractions suggested activity relationships between the broad chemical groupings. These approximated to citronellic acid ≈ guaiol > oxygenated compounds (bulnesol, eudesmols) > other components. This hypothesis was tested in Experiment 3, Appendix L, generating the data given in Table 18. Here, we compare chemical composition with activity against silverleaf whitefly, for some components and major component groups of crude extract 'C'.

Table 18: Contribution of components to activity against silverleaf whitefly (SLW).

Specimen applied at 0.2%	Composition of major components and chemical groups				Activity (% adult SLW mortality) (Appendix L)
	Citronellic acid (%)	Guaiol (%)	Bulnesol and Eudesmols (%)	Other components (%)	
C31	1	36	53	10	13, 17, 5
C32	3	2	88	7	82, 56, 70
C33	99.5	0	0	0.5	36, 30, 15
C34	75	25	0	0	32, 14, 9
C35	50	50	0	0	64, 91, 63
C36	25	75	0	0	90, 79, 100
C37	0	100	0	0	76, 81, 79

Stepwise multiple linear regression of these data uncovered equally strong positive relationships between adult whitefly mortality and citronellic acid (β = 12.7), guaiol (β = 12.5) and the oxygenated compounds group: bulnesol and the eudesmols (β = 12.9). The 'other' component group was clearly of much less significance. Citronellic acid, guaiol and the oxygenated compound group are therefore proposed for equal weighting in future batch to batch standardisation of a commercialised product.

Silverleaf whitefly responses to extract and oil application rates

There was no significant dose response of adult silverleaf whitefly, after six days on eggplant, to applied concentrations of crude extract 'C' over the range 0.1% to 0.3% (Appendix M), although phytotoxicity did increase with dose. A slight increase in mortality at 0.3% can be attributed to the greater amount of phytotoxicity at that higher dose rate.

The addition of 0.2% of a commercial paraffinic oil to these treatments gave a modest relative increase in mortality of about 10%, except for the highest level tested (0.3% crude extract 'C' + 0.2% Biopest Oil) , where a greater (relative 50%) increase in mortality associated with oil addition was attributed to the large increase in phytotoxicity observed with this combination. The phytotoxicity observed with 0.3% crude extract formulations, especially when co-formulated with 0.2% Biopest Oil, suggests that agricultural applications of formulations at this concentration are unlikely to be useful. The lowest concentration evaluated (0.1%) provided a level of control that could potentially be commercialised, particularly if used as a component of an integrated pest management (IPM) system. However, it must be recognised that only laboratory evaluation has been undertaken, and that field trials would be an essential component of any commercialisation process.

Effect of extract on a parasitoid of silverleaf whitefly

There were no significant differences in mortality to the parasitoid wasp between formulations containing 0.1% and 0.2% of crude extract 'C' (52.5% and 67.5% respectively after one day), or 0.2% and 0.4% of Biopest Oil (52.5% and 50.0% respectively) (Appendix N). However, 0.3% crude extract 'C' produced significantly greater levels of wasp mortality (76.25%), and 50 mg/L of imidacloprid produced significantly greater mortality again (100%). Although the suggested 0.1% concentration of crude extract 'C' was responsible for an appreciable amount of mortality of the beneficial wasp after one day, at 50% it was considerably less damaging than the commercial standard which, at the label rate, caused 100% mortality after one day. This property would also support the use of the crude extract in preference to the commercial standard within an IPM system, where the role of beneficial insects is valued.

Economic significance of silverleaf whitefly

An overview of the industry significance of silverleaf whitefly can be obtained at the DPI&F website - http://www2.dpi.qld.gov.au/horticultureresearch/18362.html The silverleaf whitefly (SLW), *Bemisia tabaci* biotype B was first detected in Australia in October 1994 (Gunning *et al*, 1995). It has since become a major pest of cotton and many vegetable and ornamental crops in Queensland, as detailed below. Outbreaks have also occurred in the Northern Territory, New South Wales, Western Australia and Victoria. SLW feeding can cause stunted growth, low yields and reduced quality of fruits. Crops most seriously affected by SLW to date include cotton, tomato, rockmelon, watermelon, pumpkin, squash, zucchini, cucumber, eggplant, okra, sweet potato, cabbage, cauliflower, broccoli and bean (Subramaniam *et al*, 2007a). DPI&F forecasts of gross values of production (GVP) of crops in Queensland for the 2007/08 year included $205 million (tomato), $40 million (bean), $35 million (watermelon), $35 million (rockmelon), and $50 million (cotton). Cotton production has been falling steadily for several years due to drought conditions, but GVP could double in 2008/09. In the year 2000, in each of the main vegetable production regions of Queensland, actual cultivated areas of vegetable crops suffering serious production losses due to SLW were – Bowen/Burdekin: 7520 ha; Bundaberg: 4545 ha; Lockyer Valley: 1890 ha. Growers in that year spent between $100 and $700 per

hectare on insecticides to control the pest – a total additional outlay by the industry of more than $6 million, without application costs. Medium to heavy production losses further added to the overall economic cost of the pest to industry. Between 2000 and 2004, insecticides used by the vegetable industry against SLW have included Confidor® (imidacloprid), Talstar® (bifenthrin), Chess® (pymetrozine), Admiral® (pyriproxyfen), Applaud® (buprofezin) and DCtron® (paraffinic oil) (S. Subramaniam, unpublished data). Resistance has since developed against most of these, and new chemistries are eagerly sought, particularly if less likely to suffer from development of resistance by SLW (Subramaniam *et al*, 2007b).

Other commercialisation considerations

It is intended to commence a commercialisation process for at least one agricultural application. The process is expected to involve the development by DPI&F of a Prospectus with which to seek competitive bids from potential Commercialisation Partners. It is expected that DPI&F and a selected Commercialisation Partner will complete the development of a commercial, cost effective, alternative control tool to some uses of currently registered insecticidal products, for example, the abovementioned Confidor® 200 SC. Prior to the recent development of resistance, this product was economic to use at a grower purchase cost of about $200/L. Currently, the indicated use rate of crude extract 'C' for whitefly is 0.1% which is four times the registered, previously-effective use rate for Confidor® 200 SC, suggesting that crude extract 'C' could be valued at $50/L competitively with Confidor® 200 SC (before resistance developed to the latter). Even at $50/L, at 1.7% yield on a dry matter basis, cypress sawdust at 35% moisture content would produce steam distilled oil valued at more than $500 per tonne of fresh sawdust. However, formulations containing crude extract 'C' have not yet been tested at lower use rates, raising possibilities of greater cost effectiveness. Additionally, it offers commercial opportunities as components in IPM systems, for organic production systems, and where pests have developed resistance to imidacloprid or other current active ingredients. On the other hand, as imidacloprid has recently emerged from patent protection, cheaper sources of this active ingredient and cheaper alternatives to Confidor® 200 SC are appearing in the marketplace. While this may reduce the potential selling price of a cypress extract based competitor product, it is likely to also increase the usage of imidacloprid in agriculture and consequently to increase the rate of development of resistance of agricultural pest insects. These complex interacting market-related considerations are best analysed by potential commercialisation partners as part of the intended commercialisation process. To facilitate this process, a draft Australian patent application covering a range of envisaged agricultural and veterinary pest management methods is currently being assessed by attorneys for suitability for submission to IP Australia. It will be the responsibility of the Commercialisation Partner to propose target food and/or non-food markets, develop a Commercialisation Plan, identify and meet additional data needs, and undertake the required registration process.

Conclusions

- Both alcoholic solvent extracts and steam distilled extracts of cypress milling residues contain naturally bioactive component compounds which are active against many agricultural and veterinary pests.

- The more economically produced steam distilled extract offers the best prospects for immediate agricultural pest management commercialisation. It appears to retain it's bioactivity for at least 3.5 years.
- Silverleaf whitefly is a suitable target pest for first commercialisation. Simple test formulations of the crude steam distilled extract provided useful control of silverleaf whitefly on tomato and eggplant at the lowest dose rate evaluated, *viz* 0.1%.
- Extracts can be standardised for whitefly activity by monitoring and adjusting the greatest contributing components to this bioactivity which are, with approximately equal weightings:
    - citronellic acid
    - guaiol
    - the group of eudesmol compounds and bulnesol
- There is little benefit to be gained by co-formulating the extract with paraffinic oil.

Recommendations

It is recommended that DPI&F proceed towards commercialisation of the bioactivity of cypress extractives, first focussing upon a silverleaf whitefly management application of the crude steam volatile essential oil.

Implications for Industry

Prospects appear good for commercialisation of at least one agricultural pest management application of the steam volatile essential oil from cypress sawdust, particularly if a patent can be obtained. A successful commercial product would create a demand for the essential oil that would represent a significant value-from-waste opportunity for the cypress industry. At the estimated National sawdust generation rate, potential annual crude oil production would total about 200 tonnes, potentially yielding an agricultural insecticide concentrate product worth $10 million. However, it is unlikely that the entire National sawdust output could be captured, as isolated millers generating small quantities of sawdust may not justify installation of the steam generation and sawdust distillation equipment required to realise the benefit, and relatively high freight costs of the low density sawdust are likely to make it uneconomical to move it to distant centralised extraction facilities. Most economically viable oil production situations will be large mills and groups of closely-located smaller mills.

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Appendices

Appendix A: two-spotted mites and fruit spotting bug – first screening

Appendix B: two-spotted mites – second screening

Appendix C: thrips – first screening

Appendix D: thrips – second screening

Appendix E: banana scab moth – first screening

Appendix F: silverleaf whitefly – first screening

Appendix G: silverleaf whitefly – second screening

Appendix H: adult cattle ticks – first screening

Appendix I: adult cattle ticks – second screening

Appendix J: ruminant gastrointestinal nematodes – first screening

Appendix K: ruminant gastrointestinal nematodes – second screening

Appendix L: silverleaf whitefly – third screening

Appendix M: silverleaf whitefly – evaluation with paraffinic oil additive

Appendix N: evaluation against silverleaf whitefly parasitoid

Appendix O: fungal disease organisms – first screening

Appendix A: Laboratory bioassays to evaluate cypress resin extracts against fruitspotting bug and two-spotted mite – first screening experiments

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Introduction

Fruitspotting bugs (*Amblypelta* spp.) are among the most serious and widespread pests of tropical and subtropical fruits and nuts across northern Australia (Waite *et al.*, 1993; Waite & Huwer, 1998; Waite *et al*., 2000). These bugs feed on plant tissue as both adults and nymphs, secreting a salivary enzyme which facilitates the uptake of cell contents with their sucking mouthparts. They cause production losses in essentially three ways: 1. premature fruit or nut fall; 2. scarring or blemishing of mature product, making it unmarketable or 2nd grade; and 3. damage to the vegetative growth of numerous crops. In organic production systems, examples of production losses to these bugs include 50% for macadamias, 90% for avocados and >60% for carambolas

The current annual value of all crops affected by fruitspotting bugs in Australia is in the region of $250 to $300 million. Losses to these bugs are likely to be in the order of $10-20 million pa. Organic growers currently have no effective means to control spotting bugs, apart from deploying physical barriers such as nets or bags, or introducing green tree ants as predators. Neem oil has shown some potential against these bugs but in practical terms its effects are too slow. Successful control of fruitspotting bugs in conventional farming systems has been based on broad-spectrum insecticides, and principally endosulfan. Endosulfan is still widely used in Australian horticulture but some restrictions have been placed on it (National Registration Authority, 1998). It has an uncertain future as an agricultural chemical; residues in export livestock and fish kills attributable to it have increased the pressure for deregistration.

Consumer resistance to persistent synthetic pesticides and potential residues on fruit and vegetables is growing. Environmental concerns over areas such as the Great Barrier Reef, and particularly the impact of nutrients and pesticides in runoff from adjacent farms, have highlighted the need to replace chemicals such as endosulfan with softer alternatives. The synthetic pyrethroid beta-cyfluthrin is gradually replacing endosulfan for bug control (in non-organic situations) in crops such as macadamias and avocados, but this has required a shift to crop monitoring and a limit on the number of sprays per season or the amount of crop being sprayed. Narrow spectrum compounds with activity against fruitspotting bugs are being sought for both conventional and organic farming systems, and compounds derived from cypress resin could potentially play a role.

The two-spotted mite, *Tetranychus urticae* Koch, the most significant species in a group commonly referred to as red spider mites, occurs worldwide, and has a vast range of agricultural, horticultural and ornamental hosts (including apples, beans, cotton, cucurbits, papaya, roses, strawberries and tomatoes). Feeding by spider mites on plant foliage results in yellow, mottled leaves, the undersides of which display the webbing which protects the mites and their eggs. Damage to the vegetative growth reduces the photosynthetic material, resulting in a loss in plant vigour. Losses to *Tetranychus* spp. world-wide, although impossible to estimate accurately, would run to many hundreds of millions of dollars annually. Many factors are believed to contribute to an individual plant's susceptibility to mite attack, such as nutrient levels, moisture stress, soil type, proximity to weed hosts, plant age and variety, and proximity to other crops (Brandenburg & Kennedy, 1987). However, the over-use of pesticides is considered the major factor contributing to spider mite problems in many crops, as the disruption caused to natural enemies (such as the mite-feeding coccinellid *Stethorus* and predatory mites) is often severe (Kennedy & Storer, 2000). At the same time, rapid development of resistance to miticides by spider mites is widely reported (Herron *et al.*, 1991; Herron *et al*., 1997; Ho, 2000). Any product targeting two-spotted mite needs to be resilient, soft on mite predators and pathogens and preferably of novel chemistry. Compounds produced from cypress resin may possess such attributes.

Materials and Methods

Test organisms

The fruitspotting bugs used in these bioassays were either from a colony held at DPI&F Mareeba, or collected direct from the field. The two-spotted mites were obtained from a commercial producer of predatory mites (Beneficial Bug Co., Richmond, NSW), and used within one day of receipt.

Preparation of test solutions

Three parent cypress extracts and 14 fractions were assessed during the course of this work. Stock solutions of each compound were prepared as advised by dissolving 0.5g of extract/fraction in 5 mL of acetone. Two millilitres of each 10% w/v solution was then added to a 100 mL volumetric flask, 0.2 mL of Tween 80 added and then water to make up the 100 mL mixture. All test solutions were used at 2000 mg/L, the highest rate recommended for the bioassays. Endosulfan was used as the comparative standard for fruitspotting bug at 150 mL (of 350 g/L product) per 100 L water or 525 mg/L, and abamectin for two-spotted mite at 90 mL (of 18 g/L product) per 100 L water or 16.2 mg/L.

Fruitspotting bug bioassays

One litre of each test solution or water (Control) was prepared and transferred into separate rectangular foil containers. Test solutions were agitated vigorously and then fifteen green beans immersed in them for 1 minute. The beans were then removed and allowed to dry in separate 250 mL containers for 1 hour. Three treated beans were placed in a 250 mL container for each treatment replicate. Each container also had water supplied and 1 adult male fruitspotting bug (*Amblypelta lutescens lutescens*). Cloth gauze covered the top of each container to allow airflow and so limit the effects of vaporisation. There were 5 replicates of each treatment. Room temperature was 24^{o}C throughout each experiment. Each container was checked after 24, 48 and 72 h for bug condition (dead, moribund or healthy). At the completion of the experiment, the total number of feeding sites on beans was also recorded. The cypress treatments were split across four separate test periods to facilitate handling and other demands on the insect colony.

Two-spotted mite bioassays

The bioassay method was a modification of a Petri dish technique described by Campos *et al.* (1997) and similar to that used by Park *et al.* (1996). Bean leaves were cut into squares (5 cm^2) and then immersed in the test solution or water (Control) for 1 minute, and then allowed to dry for 1 hour. Each leaf square was then moved to a 9 cm Petri dish that contained a 2 cm piece of moistened dental wick, the mites were added with a brush, and the lid sealed in place with a strip of parafilm. There were 10 two-spotted mite adults (mainly females) per replicate, and five replicates per treatment. All Petri dishes were held at 24^{o}C throughout each experiment. Experiments were checked at 24, 48 and 72 hours post-treatment to record the numbers of dead mites. The cypress treatments were split across four separate test periods because the mites needed to be checked under a microscope, which was time-consuming.

Statistical analysis of data

Percent mortality of bugs and mites was transformed to arcsine before treatments were compared by analysis of variance. For bug feeding marks and mite eggs, a square root transformation of x+1 data was undertaken before analysis. Differences between treatment means were assessed by LSD (T) tests at the 5% level.

Results and Discussion

There was 100% mortality of fruitspottting bugs in the endosulfan treatment after 72 h (Table 1) but negligible or no mortality in any cypress treatment. The number of feeding marks on beans at test completion was very variable across the treatments and statistical differences could not be established. These results suggest no contact activity of any of the cypress compounds against spotting bugs, and no clear indication of feeding deterrence. Further work with these compounds on fruitspotting bugs appears unjustified.

Table 2 shows the percentage mortality of mites under the different treatment regimes and the statistical relationships of the data. For some treatments, % mortality after 24 or 48 h was recorded to be higher than after 72 h for an individual treatment. These data can be considered anomalies caused by the difficulty in assessing mortality at 24 and 48 h because observation was restricted by the confinement of the mites within sealed Petri dishes. At 72 h the dishes were opened so that the mites could be inspected more closely, making these final data more reliable. However, in all cases the abamectin (standard) treatment had significantly higher mite mortality than any of the cypress treatments. Three of the cypress fractions (A2, A4 and C4) had significantly higher mortality of mites after 72 h than the untreated control, and this difference was supported by significantly lower numbers of eggs produced in these treatments. These fractions are possibly worth examining further for activity against two-spotted mite or other *Tetranychus* species. Miticide resistance has been an ongoing problem in the control of two-spotted mite, and new compounds will be continually required to replace or support existing chemicals. Rapid mortality on exposure to a compound is not generally a requirement of a miticide, and so the progressive mortality observed under some of the

cypress treatments should not discount their efficacy. If field assessments of the above fractions against two-spotted mite are contemplated in the future, consideration should be given to trials in strawberries (in SE Queensland). These can be conducted with greater assurance (of a result) and at less cost than an equivalent trial in papaya (in N. Queensland).

Conclusions

No cypress extract or fraction showed activity against fruitspotting bug adults. Three fractions (A2, A4 and C4) showed some activity against adults of two-spotted mite, and although not as efficacious as the abamectin standard, they could be considered for further evaluation.

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Table 1. Comparison of cypress treatments against a standard (endosulfan) and control treatments for activity against fruitspotting bugs

Treatments	***% adult bug mortality***												**Mean no. feeding marks**			
	After 24 h				**After 48 h**				**After 72 h**							
Control	0	0	0	0	0	0	0	0	0	0	0	0	5.6	4.8	2.8	4.6
Endosulfan	80	60*	0*	40*	80	100	100	80*	100	100	100	100	0.6	12.6	2.8	3.6
Crude extract 'A'	0				20				20				2.0			
A1		0				0				0				21.4		
A2		0				0				0				19.8		
A3			0				0				0				10.8	
A4			0				0				0				3.2	
Crude extract 'B'	0				0				0				1.4			
B1		0				0				0				31.0		
B2		0				0				0				17.0		
B3			0								0				4.8	
B4			0								0				18.0	
Crude extract 'A'	0								0				7.2			
C1				0				0				0				6.6
C2				0				0				0				5.4
C3				0				20				20				6.8
C4				0				0				0				2.8
C5				0				0				0				3.4
C6				0				0				0				8.4

NB. Shaded cells in the same column indicate significant difference from control at the 5% level.

* indicates some or all remaining bugs moribund

Table 2. Efficacy of cypress treatments against two-spotted mite compared to a standard (abamectin) and control treatments

Treatments	***Mean % adult mite mortality***												**Total eggs laid**			
	After 24 h				**After 48 h**				**After 72 h**							
Control	*11.4* **ab**	*6.7* **a**	*8.2* **a**	*8.7* **a**	*17.5* **a**	*12.0* **a**	*32.0* **a**	*17.9* **a**	*16.7* **a**	*20.9* **a**	*48.0* **a**	*33.8* **a**	*448* **a**	*71* **ab**	*177* **a**	*183* **a**
Abamectin	*100* **d**	*95.3* **b**	*88.6* **b**	*100* **c**	*100* **d**	*100* **c**	*98.0* **b**	*100* **b**	*98.0* **c**	*92.5* **b**	*100* **b**	*100* **c**	*5* **c**	*1* **b**	*8* **b**	*1* **d**
Crude extract 'A'			*14.2* **a**				*28.8* **a**				*36.4* **a**				*21* **b**	
A1				*32.0* **ab**				*35.5* **a**				*53.3* **ab**				*225* **ab**
A2				*48.0* **ab**				*55.0* **a**				*65.4* **b**				*42* **bcd**
A3		*23.2* **a**				*35.7* **b**				*36.7* **a**				*72* **ab**		
A4	*54.7* **c**				*56.5* **c**				*38.3* **b**				*37* **bc**			
Crude extract 'B'			*28.0* **a**				*40.4* **a**				*54.9* **a**				*47* **b**	
B1				*33.5* **ab**				*35.7* **a**				*43.5* **ab**				*60* **bcd**
B2				*28.7* **ab**				*33.6* **a**				*44.1* **ab**				*121* **abc**
B3		*18.2* **a**				*18.7* **a**				*20.9* **a**				*260* **a**		
B4	*9.2* **a**				*20.9* **ab**				*35.4* **ab**				*254* **bc**			
Crude extract 'C'				*54.0* **b**				*51.6* **a**				*54.5* **ab**				*110* **abc**
C1			*21.0* **a**				*47.4* **a**				*53.6* **a**				*60* **ab**	
C2			*24.0* **a**				*47.3* **a**				*63.3* **a**				*10* **b**	
C3		*15.2* **a**				*16.4* **a**				*32.5* **a**				*87* **ab**		
C4	*33.7* **bc**				*41.1* **bc**				*54.0* **b**				*22* **bc**			
C5		*12.9* **a**				*16.7* **a**				*34.9* **a**				*200* **a**		
C6	*21.0* **ab**				*14.0* **a**				*24.9* **ab**				*124* **b**			

NB. Shaded cells in the same column indicate significant difference from the control at the 5% level. Significant differences between treatments in the same column are indicated by different letters.

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Appendix B: Laboratory bioassays to further evaluate cypress extracts against two-spotted mite, *Tetranychus urticae* – second screening

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Introduction

An initial series of laboratory bioassays was conducted in 2005 to evaluate a number of cypress resin extracts against the two-spotted mite, *Tetranychus urticae* Koch. This mite is a global pest of a vast range of agricultural and horticultural crops, and insecticide resistance is a major factor in its effective control. Of the three parent and 14 fractions tested initially, three of the fractions (A2, A4 and C4) showed some activity against mite adults. Significant reductions in the number of eggs produced under these treatments supported the mortality data. Although not as efficacious as the abamectin standard, these treatments indicated sufficient activity to warrant further investigation. As a consequence, a further series of bioassays was conducted on a number of compounds based on these findings.

Materials and Methods

Test organisms

The two-spotted mites were obtained from a commercial producer of predatory mites (Beneficial Bug Co., Richmond, NSW), and used within three days of receipt.

Preparation of test solutions

Three full cypress extracts, 8 fractions and one other compound were assessed during the course of this work. Stock solutions of each compound were prepared as advised by dissolving 0.5 g of extract/fraction in 5 mL of acetone. Two millilitres of each 10% w/v solution was then added to a 100 mL volumetric flask, 0.2 mL of Tween 80 added and then water to make up the 100 mL mixture. All test solutions were used at 2000 mg/L, the highest rate recommended for the bioassays. Abamectin was used as the comparative standard at 90 mL (of 18 g/L product) per 100 L water or 16.2 mg/L a.i.

Two-spotted mite bioassays

The bioassay method was a modification of a Petri dish technique described by Campos *et al.* (1997) and similar to that used by Park *et al.* (1996). Bean leaves were cut into squares (5 cm^2) and then immersed in the test solution or water (Control) for 1 minute. They were then allowed to dry for 1 hour. Each leaf square was then moved to a 9 cm Petri dish that contained a 2 cm piece of moistened dental wick, the mites were added with a brush, and the lid sealed in place with a strip of parafilm. There were 10 two-spotted mite adults (mostly females) per replicate, and five replicates per treatment. All Petri dishes were held at 24^{o}C throughout each experiment. Experiments were checked at 24, 48 and 72 hours post-treatment to record the numbers of dead mites. The cypress treatments were split across two separate test periods because the mites needed to be checked under a microscope, which was time-consuming.

Statistical analysis of data

Percent mortality of mites was transformed to arcsine before treatments were compared by analysis of variance. For mite eggs, a square root transformation of x+1 data was undertaken before analysis. Differences between treatment means were assessed by LSD (T) tests at the 5% level.

Results and Discussion

Table 1 shows the percentage mortality of mites under the different treatment regimes and the statistical relationships of the data. For some treatments, % mortality after 24 or 48 h was recorded to be higher than after 72 h for an individual treatment. These data can be considered anomalies caused by the difficulty in assessing mortality at 24 and 48 h because observation was restricted by the confinement of the mites within sealed Petri dishes. At 72 h the dishes were opened so that the mites could be inspected more closely, making these final data more reliable. In all cases, the abamectin (standard) treatment had significantly higher mite mortality than any of the cypress treatments after 72 hours. However, the number of eggs laid in all the A treatments and in C12 were not significantly different from the number laid in the abamectin treatment. There were no statistical differences in efficacy between any of the A treatments. However, the overall data suggest that the full A extract and A12 are possibly worth examining further for activity against two-spotted mite or other *Tetranychus* species. There was some indication of plant damage by the full A extract, but this would have to be verified on living plants. If field assessments of the above fractions against two-spotted mite are contemplated in the future, consideration should be given to trials in strawberries (in SE Queensland). These can be conducted with greater assurance (of a result) and at less

cost than an equivalent trial in papaya (in N. Queensland). An alternative to both would be glasshouse trials with tomato or bean plants.

Table 1. Efficacy of cypress treatments against two-spotted mite compared to a standard (abamectin) and control treatments

Treatments	*Mean % adult mite mortality*						**Total eggs laid**	
	After 24 h		**After 48 h**		**After 72 h**			
Control	*9.9* **e**	*2.9* **d**	*23.1* **c**	*11.7* **c**	*37.1* **c**	*15.9* **c**	*514* **a**	*535* **a**
Abamectin	*100* **a**	*98.0* **a**	*100* **a**	*100* **a**	*97.8* **a**	*95.6* **a**	*1* **b**	*10* **c**
Crude extract 'A'	*93.3* **ab**		*89.3* **ab**		*72.9* **b**		*6* **b**	
A11	*40.2* **d**		*58.4* **bc**		*52.8* **bc**		*43* **b**	
A12	*68.0* **c**		*86.7* **ab**		*66.9* **b**		*3* **b**	
A13	*84.3* **abc**		*61.1* **bc**		*68.3* **b**		*45* **b**	
A14	*66.7* **cd**		*75.8* **ab**		*66.5* **bc**		*18* **b**	
A15	*81.8* **bc**		*65.1* **bc**		*75.6* **b**		*49* **b**	
Crude extract 'B'		*35.5* **b**		*46.3* **b**		*30.7* **bc**		*254* **b**
Crude extract 'C'		*27.5* **bc**		*37.9* **b**		*39.3* **b**		*170* **b**
C12		*43.7* **b**		*46.3* **b**		*26.3* **bc**		*78* **bc**
C13A		*21.8* **bc**		*43.3* **b**		*34.2* **b**		*196* **b**
C14		*26.7* **bc**		*34.4* **b**		*49.8* **b**		*176* **b**
C5		*16.4* **cd**		*30.1* **bc**		*17.0* **c**		*159* **b**

NB. Shaded cells in the same column indicate no significant difference from the standard (abamectin) at the 5% level. Significant differences between treatments in the same column are indicated by different letters.

Conclusions

Crude extract 'A' and the A12 fraction could be considered for further work against *Tetranychus* species, as they were particularly effective at suppressing egg production during the bioassays.

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Appendix C: A laboratory evaluation of cypress extract fractions against the thrips in a stand of field lupins – first screening

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Introduction

Thrips species are important plant pests not only for the direct damage that is inflicted (e.g. stunting, malformation of plant parts, fruit spotting) but also as effective vectors of plant virus diseases.

Western flower thrips, *Frankliniella occidentalis,* (Pergande) is such a pest. Not only is it an efficient vector of tomato spotted wilt virus (Lewis, 1973) but it directly blemishes apples and stone fruit, with severity depending upon cultivar, (Page and Nimmo, 2001).

Although some thrips may assist the pollination of lupins, (Annand, 1926) the presence of *F. occidentalis* in lupin cover crops in deciduous orchards allows effective overwintering and rapid build-up of pest thrips populations to infest susceptible orchards in the consequent spring, (Kirk and Terrry, 2003).

There is only one chemical currently registered for *F. occidentalis* in lupins.(Department Primary Industries and Fisheries 2005) while *F. occidentalis* has shown an exquisite propensity to rapidly develop resistance to many insecticides. As part of the overall management strategy for the pest, specific rotation of insecticide groupings as a means of chemical control, is fundamental. However in many affected crops there are few such groupings to rotate into.

Newer chemistry, such as the cypress extracts, with any potential thripicidal action, may help to redress this imbalance in available chemical groups.

Materials and methods

Test organisms

The thrips used were of varied age, sex and probably species. Both immature and adult forms were found and no attempt was made to identify immatures.

A selection of adults were collected in 70% ethanol to allow for subsequent slide mounting, clearing and consequent microscopic identification. They are presently in the hands of a specialist to determine species.

Superficial examination under a stereo microscope would suggest that the bulk of the species would be western flower thrips (*Frankliniella occidentalis*) .

Test solutions

With increasing cold weather militating against the survival of the thrips population on the lupin field, the numbers of tested extracts was reduced by the removal of the A, B and C parental lines. It was considered that insufficient thrips might be present to allow the five replications, if adverse conditions e.g. plants hit by frost, prevailed.

From each of the 14 fractions, 0.5 g of material was weighed out and dissolved in 5 mL acetone (technical grade). One millilitre of this solution was further diluted with tap water in a 50 mL volumetric flask after 0.1 mL of non-ionic wetter (Agral ® 600) was added. Tap water and Agral® were similarly used for the nil treatment.

All the dippings used these solutions. After each day's dippings the material was stored in a refrigerator running at 5–8°C. After storage, the material was allowed to adjust to ambient temperature by placing on the laboratory bench for 2–3 hours before reusing.

Procedure with the test solutions

A collection of narrow leaved lupin (*Lupinus angustifolius*) inflorescences was taken at random from a small field planting at Applethorpe Research Station, each day from 20–23 June 2005 and once again on 27 June 2005. Each time period constituted a replicate and was analysed (Anova, LSD) accordingly.

The more open florets at the base of each inflorescence were stripped from the flower and bulked before being distributed equally into 16 twenty-five ml plastic portion cups. The first ten florets from each cup were dissected under a stereomicroscope and the numbers of thrips counted. Dead thrips were counted separately from live thrips when no movement of any appendage was observed.

The remaining florets were individually immersed in the 14 extract solutions as well as the dimetoate and water only treatments; for 3 seconds with fine watchmakers forceps. The forceps allowed the forcing of the petals apart and allowed better ingress of solutions. Florets were air dried on paper towelling before being placed on moistened filter paper in 90mm petri dishes. The petri dishes were then incubated for 24hrs at 20°C in a constant temperature incubator before being destructively examined for thrips.

Results and Discussion

The results of the dippings are shown in Table 1. No pre-treatment comparison can be made directly to the counts of thrips that were present on the actual florets dipped in the treatments, by virtue of the destructive nature of the sampling. However the untreated set counts give a good approximation of any uneven distribution between treatment sets. The pattern of thrips incidence in the untreated set indicates a degree of uniformity between the treatments with no significant differences at the 5% level for both dead thrips and live thrips respectively.

Table 1: Numbers of thrips (mainly *Frankliniella occidentalis*) surviving on narrow leaved lupin florets after dipping in a range of solutions and incubation for 24hrs at 20°C.

Numbers of Thrips per Floret				
Treatment	**Untreated set**		**Treated set**	
	No. Dead	**No. Live**	**No. Dead**	**No. Live**
Water + wetter	0.08	1.64	0.09 bc	2.26 ab
A1	0.06	1.74	0.13 bc	1.74 abc
A2	0.06	0.08	0.15 bc	2.39 a
A3	0.06	2.10	0.20 b	1.64 abcd
A4	0.08	1.58	0.14 bc	1.53 bcd
B1	0.02	1.98	0.16 bc	1.73 abc
B2	0.02	1.28	0.11 bc	1.49 bcd
B3	0.32	1.40	0.04 c	1.76 abc
B4	0.1	1.60	0.14 bc	1.55 bcd
C1	0.04	2.32	0.18 bc	1.02 cde
C2	0.04	1.56	0.09 bc	1.20 cde
C3	0.06	1.84	0.11 bc	1.20 cde
C4	0.04	1.62	0.17 bc	0.98 de
C5	0.06	1.82	0.15 bc	1.38 cd
C6	0.1	2.06	0.70 a	0.90 de
Dimethoate	0.06	1.70	0.81 a	0.51 e
	n.s.	n.s.		

Values, within columns, with a common letter do not differ at the P = 0.5 level of probability
n.s.. = non significant F

In the treated set, the dimethoate standard gave relatively poor control. However the C fractions, with the exception of C5, gave control equivalent to the standard with the trend favouring both C6 and C4. Notwithstanding its lack of equivalence with dimethoate, C5 was significantly better than the water + wetter treatment. All of the other treatments gave similar control to the water + wetter standard.

Dimethoate was chosen as a standard because it was the only insecticide registered for thrips in lupins, (Department of Primary Industries and Fisheries 2005). Moreover only low levels of resistance to dimethoate had been previously detected for *F. occidentalis* (Herron and James 2003) from this location.

However the method of use did not allow dimethoate and possibly the extract fractions, to show their systemic ability (Thomson, 1995) fully. The necessity to dip florets rather than allow more direct contact with the active solutions is another disadvantage of the method used. The generally lower mortality may reflect this lack of contact. In addition, although more mortality may have occurred beyond the 24hrs of incubation, decay of the floral parts was marked after 24hrs, necessitating the use of this time interval.

Conclusions

The crude 'C' extract would warrant further trialling against thrips species. If the constraints of this trial could be overcome – an extremely short timeframe in one of the coldest periods of the year – by allowing more time to utilise better trial design; clearer outcomes may ensue.

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Appendix D: A laboratory evaluation of some cypress pine extracts against thrips in a native shrub, wild rosemary, *Cassinia quinquefaria* (second screening)

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Introduction

An earlier study using the field infestation of thrips in a stand of narrow leaved lupin (*Lupinus augustifolius*) growing in a field of the Applethorpe Research Station had shown that some of the cypress extract fractions, especially the 'C' fractions were effective in reducing populations of thrips. The dominant thrips in this instance were Western flower thrips, *Frankliniella occidentalis* , onion thrips, *Thrips tabaci* , and tomato thrips, *Thrips schultezi.* (Kennedy *et al*. 2005)

It was hoped to use the populations of thrips infesting apple blossom for a consequent test of the extracts, where the populations were expected to also include another important pest species, plague thrips, *Thrips imaginis.* However testing the extracts was delayed until after flowering.

As most of the pest species are quite catholic in their hosts, as long as the hosts are in blossom; (Lloyd 1973, Chellami et al 1994, Pearsall & Myers 2000, Broughton et al 2004, Atakan & Uygur 2005, Mound 2005, Steiner & Goodwin 2005) it could be expected that wild hosts blooming consequent to the period of apple bloom, would also carry varying loads of thrips. Because of the need for a large quantity of blossom, availability of such volumes was considered along with the capacity of the species to carry a load of thrips.

Materials and methods

Test organisms

Collection of the floral parts of six plant species (in bloom for the period 28th February 21st March 2006) were made from vegetation growing alongside apple orchards in the district. Three collections were made, at weekly intervals and the thrips load in each sample extracted by heat using an enclosed battery of nine Tolgren funnels, on loan from the Fire Ant Control Centre, Wacol. Extractions were made into 70% ethanol and the numbers of thrips both mature and immature, counted under a stereomicroscope.

The samples were subjected to heat from nine 60 watt incandescent bulbs, and the dried weight of each sample recorded. A subsample of the thrips in each extraction was taken for consequent clearing and mounting for microscopic examination and species determination. Initial determinations would suggest that *T. imaginis* was also present.

Procedure with the test solutions

0.5 g of each material was diluted in 5 mL acetone (technical grade) before one millilitre of this solution was further diluted with tap water and the non-ionic wetter Agral 600 in a 50 mL volumetric flask. Bayer's Lebaycid 550 g fenthion per L was used as a control standard, at the recommended rate for thrips, 0.039% a.i. (rate of 5 mL product per 7L water)

Ten open inflorescences of wild rosemary were held in a narrow necked Erlenmeyer flask, containing water and acting as a vase. Each treatment was applied to the inflorescences by a Pistache™ air brush connected to a small air compressor. This procedure was repeated on five separate weekly occasions from the 20th March – 22nd April, 2006. Each weekly interval was considered a replicate. The sprayed samples were held on a laboratory bench for 3 further days before extraction in the Tolgren funnels overnight, as outlined above. The rationale being that if thrips had been killed by the spray, they would be unable to exit the bloom and crawl away from the light and heat of the lamp. If they were repelled they would have gone before extraction. Internal laboratory temperatures were recorded by max-min thermometer and did not vary between the range of 19–26ºC.

Numbers of thrips per sample and the weight of the dry material was recorded. Data were analysed by ANOVA and LSD using the Genstat® programme.

Results and Discussion

The results of the comparison of plant hosts is shown in Table 1.

Table 1: Thrips load carried by a range of plant hosts growing adjacent to apple orchards on the Granite Belt.

Host names		No. thrips / sample	*No. thrips/ g dry weight of sample*
Wild rosemary	*Cassinia quinquefaria*	18a	6.2a
Veined verbena	*Verbena rigida*	44ab	8.0ab
White clover	*Trifolium repens*	47ab	38.1c
Purple top	*Verbena bonarienis*	95ab	11.6ab
Coreopsis	*Coreopsis lanceolata*	111ab	47.4c
Wild turnip	*Rapistrum rugosum*	684c	120d

Values, within columns, followed by a common letter do not differ at the P=0.05 level of probability.

Although there are clear differences between the thrips loads carried by different plant hosts, the abundance of *C. quinquefaria* blossom at the time, made it the host of choice.

The results of the spraying trial on wild rosemary is shown in table 2.

Table 2: Effect of sprayed cypress pine extracts or fenthion on thrips load of blossoms of wild rosemary

Treatment	No. thrips / sample			No. thrips / g dry wt of sample		
	Pretreatment	3DAT	Transformed*	Pretreatment	3DAT	Transformed*
Fenthion	27.7a	2.0a	1.55a	3.77a	0.23a	0.85a
Ext. 'A'	26.3a	9.0ab	2.82ab	3.46a	1.02ab	1.18ab
Ext. 'B'	29.3a	7.4ab	2.77ab	3.42a	1.16ab	1.26ab
Ext. 'C'	26.3a	9.6ab	2.95bc	3.18a	1.34ab	1.30ab
C12	29.7a	13.0bc	3.50bc	3.65a	1.64ab	1.42b
C13A	34.7a	7.4ab	2.59ab	5.22a	1.02ab	1.22ab
C13B	33.7a	10.6abc	2.76ab	3.17a	1.34ab	1.20ab
C14	34.3a	13.4bc	3.50bc	3.68a	2.46bc	1.59bc
C15	38.0a	8.2ab	2.76ab	4.03a	1.08ab	1.21ab
Nil (control)	26.0a	18.2c	4.28c	3.03a	3.46c	1.95c

***** √x+0.5 transformation Shaded cells in the same column indicate no significant difference from the standard (fenthion) at the 5% level.

With the possible exception of C14, all treatments appeared to have a significant effect on thrips numbers. However, although the treatments were not significanly different from the standard fenthion at 0.039%, the trend did favour this standard insecticide.

Conclusions

The trial would suggest that many of the 'C' fractions as well as 'A' and 'B' have a thripicidal action. The trial was unable to separate mortality from any repulsion that may also have occurred.

This lack of finesse is a feature of having to rely on wild caught material when dealing with small quantities of active. Most of the insecticide companies have stock colonies in-house to directly test small quantities of new chemistry in their biological screening. Because of the vagaries of field caught material and the small quantity of active available, it is recommend that consideration be given to directing the more promising active fractions in that direction.

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Appendix E: Evaluation of 17 Cypress Extracts for Insecticidal Activity against Banana Scab Moth larvae - *Nacoleia octasema* (Meyrick) (Lepidoptera: Pyralidae)

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Abstract

Extracts from cypress pine (3 parents and 14 fractions) were evaluated against 5th instar banana scab moth larvae for their insecticidal properties. All cypress treatments were not significantly different ($p>0.05$) when comparing mortality to the control treatment in ingestion residue assays and were significantly less effective when compared to chlorpyrifos. Repellency was observed in the cypress treatments A1, B0, B4 and C5. Fruit damage was all under 5% and not significantly different to the industry standard insecticide and control treatments.

Introduction

Natural plant extracts as biocides or insect-control agents can be traced back at least as far as the Romans and Chinese who used plant species such as white hellebore (*Veratrum album*) and black hellebore (*Veratrum nigrum*) to control agricultural pests (Gbolade, 2001). Nicotine (from tobacco - *Nicotiana tabacum*) and rotenone (from *Derris* and *Lonchocarpus*) are other examples of natural compounds used to control agricultural pests. New successes in this field are the azadiractins (from the neem tree - *Azadirachta indica*), which have hormonal, anti-feedant, and repellency activity against many insect species. Essential oils and plant extracts are an important natural resource of pesticides/insectides (Raguraman and Singh, 1997) or larvicides (Jacobson, 1983; Adebayo *et al.*, 1999; and Murty Jamil, 1987); or insect repellents (Sadik, 1973; Thorsell *et al.*, 1998 and Oyedele *et al.*, 2000;). Sadik lists the essential oils of cedarwood, citronella, eucalyptus, pennyroyal, turpentine and wintergreen as having traditional uses for their insect repellent properties. Arroyo, 1995, reports that natural plant extracts are an under exploited resource in pest management and should be further investigated.

The banana scab moth (BSM) is a major pest of bananas. Its distribution covers Malaysia, Singapore, Indonesia, northern Australia and the island groups of the southwest pacific and Malay Archipelago (Paine 1964). The pink to brown larvae feed on developing fruit scarring the surface and range in length from 1.5 mm at egg emergence to 25 mm when fully developed (Pinese and Piper 1994). If left uncontrolled the larvae can destroy up to 100% of developing fruit. Management of this pest in Australia is primarily by the use of synthetic insecticides applied as an injection to new emerging banana bells followed by a bunch spray 14 days later. Unfortunately, many of the insecticides used to control this pest are toxic to the environment and human health and are not compatible with environmental management systems (Astridge 2004).

Research based on natural products is increasingly important because it has implications in integrated and biological pest management in agriculture. Plant secondary metabolites play an important role in plant–insect interactions and therefore such compounds may be beneficial in developing pest management systems (Camps, 1988). Sampling the native flora might also lead to the discovery of new compounds suitable for managing plant pests (Cuñat *et al.*, 1990). This paper reports the results of screening different cypress pine compounds and fractions for insecticidal activity against 5th instar BSM larvae.

Methods

Ingestion and residual bioassays were conducted on 5th instar BSM larvae. 17 treatments were compared to a control and an industry standard insecticide. Each of the cypress treatments was prepared to make 10% w/v stock solutions. The final solutions (100mL) of 2% ethanol, 0.2% Tween 80 and 0.2% cypress extract or fraction. The industry standard insecticide (ISI) was chlorpyrifos (Lorsban® 750 WG) at the label rate (0.125%). The control treatment was water and Tween 80 (0.2%). Chemical free immature banana bunches were collected 10 days after bell emergence from an orchard at the CWTA research station at South Johnstone, Queensland. A total of 760 5th instar BSM larvae were harvested from a lab culture and placed into petri dishes before being placed on treated fruit. One fruit hand (six fruit) per replicate was sprayed to the point of runoff for each treatment and allowed to dry on newspaper for 20 minutes before being placed in 750 mL polyethylene takeaway food containers. Each takeaway container had perforated holes in the lids to allow air movement. Ten larvae were placed onto the treated fruit inside these containers and replicated four times for each treatment. All treatments were placed in a constant temperature (CT) room using a RCB trial design blocking for uneven temperature, light and air movement. CT room settings were

27°C, 80% RH and a 14:10 day: night photoperiod. Mortality was assessed at 1, 3,7 and 10 days after treatment. At the end of the trial, fruit assessments were carried out to examine the level of damage caused by the larvae. The data were arcsine transformed prior to conducting ANOVAs, which compared mean mortality levels of the cypress treatments to the ISI and control and the mean feeding damage for each treatment.

Results and Discussion

Larval Mortality

All cypress treatments achieved low levels of larval mortality and were not significantly different ($p>0.05$) when compared to the control treatment. The industry standard insecticide was significantly superior ($p<0.05$) to the cypress treatments achieving 100% mortality 3 days after treatment (Table 1). Most of the larvae effectively reached pupation in all cypress treatments. The results of this ingestion assay show that cypress extracts are not effective in achieving mortality of 5th instar BSM larvae at the rates tested when compared to the industry standard insecticide.

Table 1: Results of cypress extract insecticide trial showing the mean levels of scab moth mortality for 17 cypress treatments compared to the industry standard insecticide and control.

Treatment	% mortality Day 1	% mortality Day 2-3	% mortality Day 4-7†	Accumulative % mortality Day 1-3	Accumulative % mortality Day 1-10
Control	0.6 a*	0.6 a	0	2.6 ab	2.6 ab
Crude 'A'	0 a	2.6 a	0	2.6 ab	2.6 ab
A1	0 a	0 a	0	0 a	0 a
A2	0.6 a	5.7 a	0	7.5 ab	7.5 ab
A3	0.6 a	0.6 a	0	2.6 ab	2.6 ab
A4	0.6 a	5.7 a	0	10.0 b	10.0 b
Crude 'B'	0 a	0 a	0	0 a	0 a
B1	3.8 a	0.6 a	0	7.5 ab	7.5 ab
B2	0 a	2.6 a	0	2.6 ab	2.6 ab
B3	1.3 a	0 a	0	1.3 ab	1.3 ab
B4	2.6 a	2.6 a	0.6	7.5 ab	9.4 b
Crude 'C'	0 a	0.6 a	0	0.6 ab	0.6 ab
C1	0.6 a	7.5 a	0	9.4 b	9.4 b
C2	0.6 a	0 a	0	0.6 ab	0.6 ab
C3	1.3 a	0.6 a	0	3.8 ab	3.8 ab
C4	2.6 a	0.6 a	0	5.7 ab	5.7 ab
C5	1.3 a	6.2 a	0.6	8.8 ab	10.4 b
C6	2.1 a	1.3 a	0	3.8 ab	3.8 ab
**ISI	92.5 b	7.5 a	0	100.0 c	100.0 c

* Means followed by a similar letter do not differ significantly ($p>0.05$)

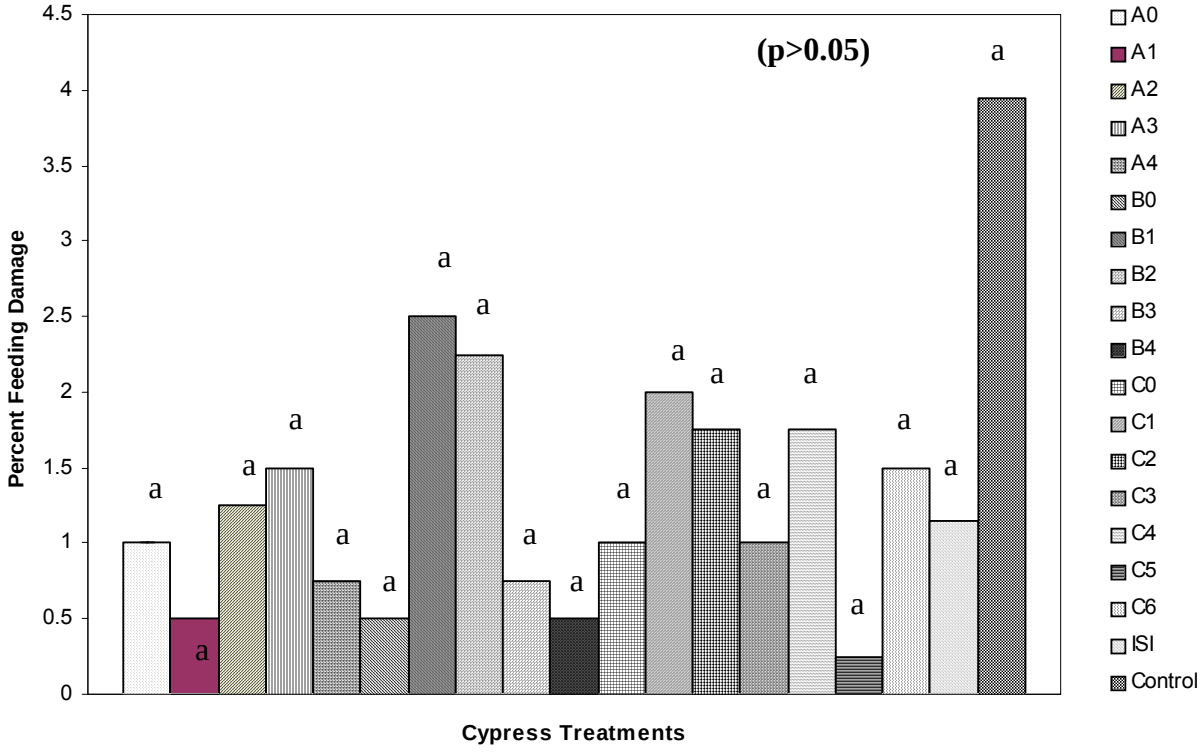
† Only 2 non-zero data values so this data set did not warrant analysis

Mortality figures were arcsine transformed prior to analysis, and the figures presented are back-transformed percentages. **Treatment ISI refers to an industry standard insecticide.

Fruit Damage

Crude extract 'B' and fractions A1, B4 and C5 showed a high level of repellency to BSM larvae. Many of the caterpillars in these treatments moved off the fruit and stayed on the container walls and would not feed on the fruit. There were no significant differences ($p>0.05$) between the level of fruit damage for all treatments when compared to the ISI and control treatments. Although not significantly different, the level of fruit scarring caused by larvae was appreciably lower in the cypress and ISI treatments (Figure 1). The low level of fruit damage in the control treatment may have been caused by the quick development of 5th instar larvae to pupation, which takes around 7–9 days at 27ºC.

Figure 1. Mean levels of BSM fruit damage after treatment with cypress extracts compared to chlorpyrifos, an industry standard insecticide (ISI) and control treatments. Means followed by a similar letter do not differ significantly (p>0.05).



Conclusion

The cypress extracts showed low levels of mortality at the rates tested but the low levels of fruit damage and the repellency activity of the cypress treatments may be worth further investigating at higher rates and earlier larval stages.

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Appendix F: Effectiveness of Cypress extracts against Silverleaf whitefly (*Bemisia tabaci)* on Tomato – first screening

Researchers Siva Subramaniam, and Verni Sivasubramaniam, DPI&F, Bowen, Queensland

Indroduction

Silverleaf Whitefly (SLW), *Bemisia tabaci* Biotype B, also known as *Bemisia argentifoli*, is a serious pest of many vegetable crops in Queensland. This polyphagous pest cause severe economic damage through direct feeding and injecting toxic saliva into the plants which leads to stunted growth, low-quality fruit and honeydew contamination on fruits.

SLW has demonstrated an outstanding capacity for developing high level of resistance to many insecticides being used by growers. Public concern over the hazards associated with the synthetic insecticides, and the rapid resistance development within insect populations against the synthetic products has evoked interest on alternative pest control strategies. Plant origin products, as alternative to conventional insecticides, have potential benefit in IPM programs.

The objective of this study was to evaluate the efficacy of cypress extracts against silverleaf whitefly life stages (adults, eggs and nymphs) under laboratory conditions.

Materials and Methods

In this experiment, a total of sixteen extracts with an untreated control were assessed against SLW adults, oviposition and nymph development.

Tomato seedlings (variety Guardian) were grown in insect proof cages to avoid SLW infestation. When the seedlings were in the 5-week old stage, they were trimmed, leaving only four young leaves, and planted individually in 12 cm plastic plots.

For each treatment, 0.5 g of cypress extract was dissolved in 5.0 mL ethanol, and then 2 mL of the dissolved extract was mixed with 0.2 mL of non-ionic surfactant (Wetter 600®) and 98 mL of distilled water to produce 0.2% spray solution. The surfactant (0.2 mL) was added to distilled water which served as the control. Approximately 15 mL of the spray solution was sprayed to each plant (replicate) with a hand-held mister and allowed to dry for 3–4 hrs.

Around 25 whitefly adults were introduced to each treated plant, which was covered with a cylindrical film cage (Photo 1). All the 17 treatments were arranged in a randomised block design with four replicates in a controlled temperature cabinet where the temperatures were programmed at four different set-points (20, 23, 25 and 27°C) and with 60% relative humidity and photoperiod of 14:10 LD.

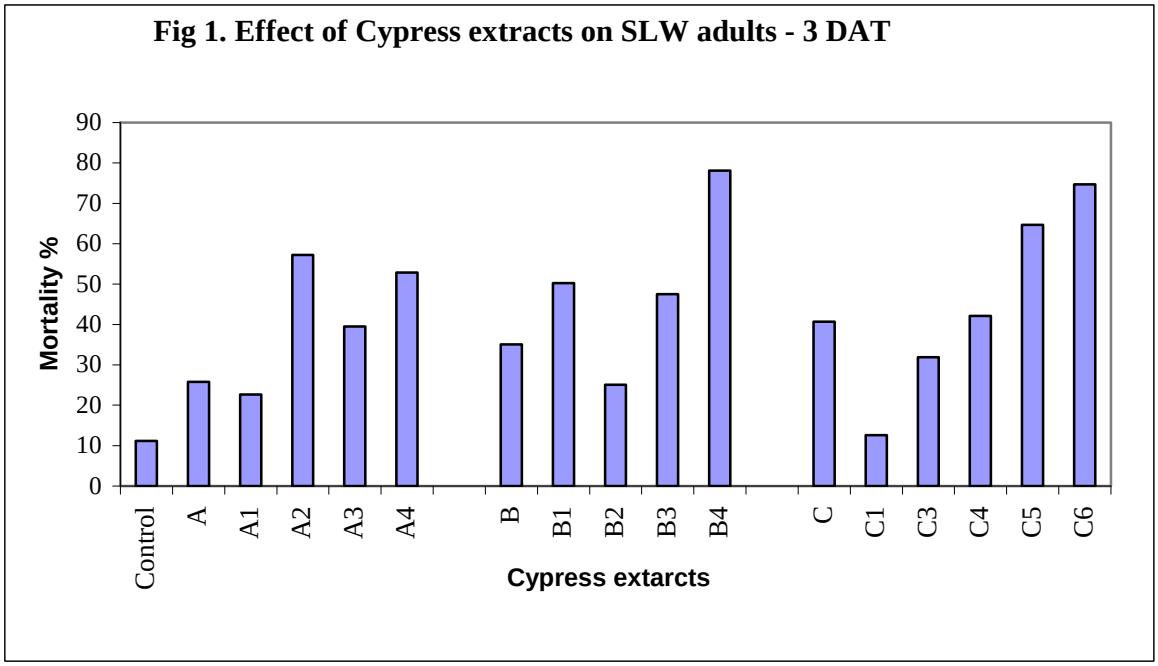
Adult mortality was recorded 4 and 7 days after treatment (DAT). Four leaflets were collected randomly from each plant at 14 and 28 DAT. The leaflets were assessed under microscope to record hatched and unhatched eggs, small nymphs and large nymphs.

Photo 1. Cylindrical film cage set-up used to assess adult mortality

Results and Discussion

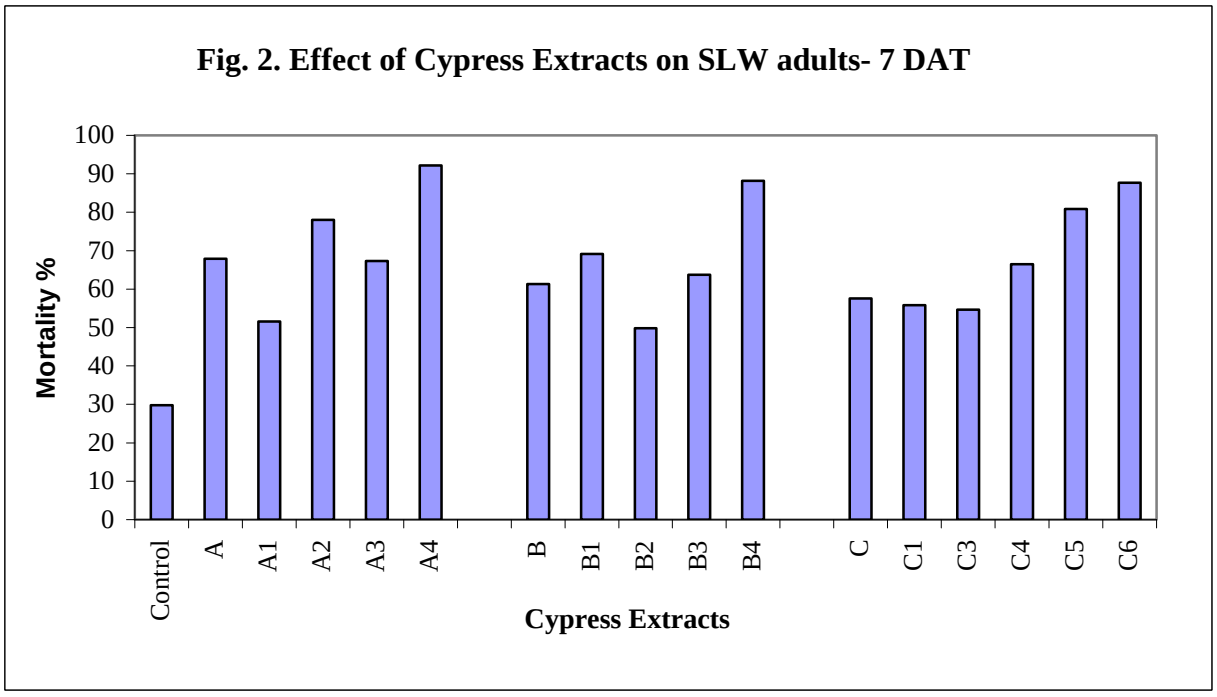
Effect on adult mortality

The effects of cypress extracts on SLW adult mortality are shown in Figures 1 and 2 and Table 1.



Among the cypress extracts and fractions tested at 7 DAT, A2, A4, B4, C5 and C6 showed high levels of adult mortality (78–92%) (Fig. 2). High adult mortality was recorded in the treatments B4 and C6 within 4 days after treatment (Figure 1), indicating these fractions may have a quick knock-down effect on adults.

The crude extracts and fractions A1, B2, C1 and C3 did not show a significant ($P<0.5$) level of adult mortality compared with the untreated control at 7 DAT. The treatments B1, B3 and C4 showed an intermediate level of mortality (63–69%), however these compounds were less worthy of consideration for further studies because of their delayed mortality effect on adults (Figure 1).



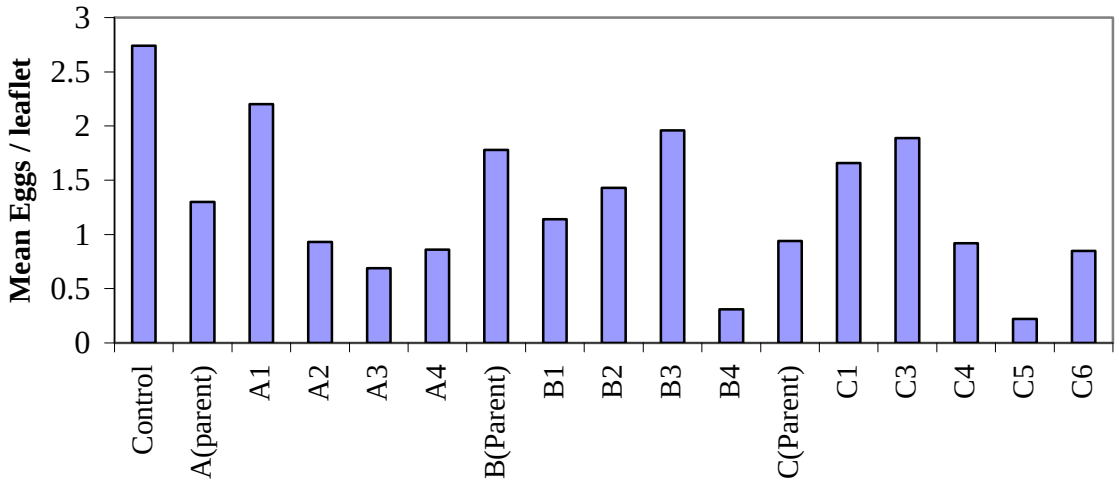
Effect on oviposition and nymph development

The effect of cypress extracts on oviposition, egg hatchability and nymph development is shown in Figures 3, 4 and 5. The data are summarised in Table 2.

The treatments B4 and C5 had significantly (P> 0.05) fewer eggs than other treatments (Figure 3). The egg densities in the B4 and C5 were 89 and 92% lower than in the untreated control, respectively. This may be due to the high level of adult mortality that occurred in these treatments within four days.

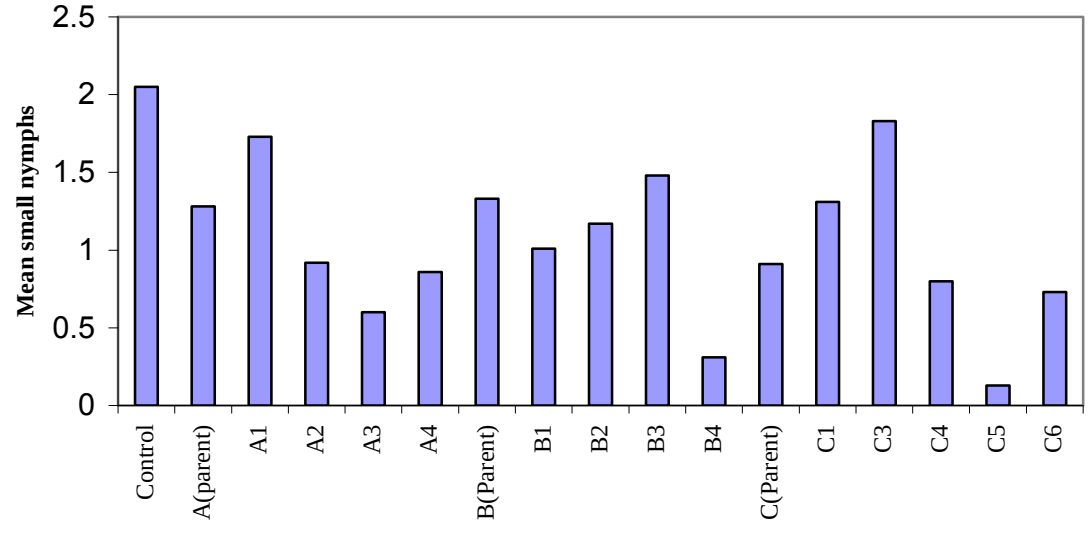
The treatments A, A1, B, B1 B2, B3, C1 and C3 did not significantly reduce the egg numbers compared with control. The adult survival rate was higher in these treatments that this may have increased egg deposition on these treatments.

Fig. 3 Effect of cypress extracts on SLW oviposition



Out of these 16 extracts A3, A4, B4 and C5 treatments produced significantly fewer nymphs than the control at both leaf sampling dates (14 and 28 days after treatment). The results indicated that the extracts B4 and C5 caused some impact on egg hatchability and nymph establishment (Figure 4). In this experiment we did not intend to study the toxicological effect of extracts on egg hatchability or nymph developments. However, such a study is essential to further explore potential of these extracts on SLW life stages.

Fig 4. Effect of cypress extracts on egg hatchability



Conclusions

Five cypress fractions (A3, A4, B4, C5 and C6) caused significant adult mortality when applied at a rate of 0.2%. A subsequent reduction in egg densities was also clearly seen.

Overall, the results show that the extracts B4, C5 and C6 have insecticidal properties, which can cause adult mortality, reduce oviposition and interfere in nymph developments. However, further studies are required to investigate this in more details to confirm the findings.

Table 1. Effect of cypress extracts on silverleaf whitefly adult mortality.

Treatment	**Adult Mortality (%)**	
	4 DAT	**7 DAT (**Cumulative)
Control	11.2 a	29.8
A (parent)	25.8 a	67.9
A1	22.6 a	51.6
A2	57.2	78.0
A3	39.5	67.3
A4	52.9	92.2
B (parent)	35.1	61.3
B1	50.3	69.1
B2	25.1 a	49.8
B3	47.5	63.7
B4	**78.1**	88.2
C (parent)	40.7	57.6
C1	12.6 a	55.8
C3	31.9 a	54.7
C4	42.1	66.5
C5	64.7	80.9
C6	74.7	87.7

Table 2: Effect of cypress extracts on SLW oviposition, egg hatchability and nymph establishments

Treatment	Mean eggs/ leaflet	Mean eggs hatched	Mean Nymphs established
Control	2.74	2.05	1.57
A (parent)	1.3	1.28	0.73
A1	2.2	1.73	0.59
A2	0.93	0.92	0.39
A3	0.69	0.6	0.19
A4	0.86	0.86	0.24
B (parent)	1.78	1.33	1.31
B1	1.14	1.01	0.75
B2	1.43	1.17	1.0
B3	1.96	1.48	1.05
B4	0.31	0.31	0.13
C (parent)	0.94	0.91	0.87
C1	1.66	1.31	1.58
C3	1.89	1.83	1.33
C4	0.92	0.80	0.67
C5	0.22	0.13	0.44
C6	0.85	0.73	0.59

Appendix G: Second screening of Cypress extracts against Silverleaf whitefly (*Bemisia tabaci)* on Tomatoes

Researchers Siva Subramaniam, and Verni Sivasubramaniam, DPI&F, Bowen, Queensland

Introduction

The silverleaf whitefly (SLW), *Bemisia tabaci* biotype B was first detected in Australia in October 1994. It is a major pest of vegetable, cotton and ornamental crops. SLW feeding can cause stunted growth, low yields and reduced quality of fruits. In some hosts, feeding can induce physiological disorders such as squash silverleaf, uneven ripening in tomatoes and white stem in broccoli. They also secrete large quantities of very sticky honeydew.

In 2000, SLW outbreaks in the coastal vegetable production areas led to more than $6 million of additional pesticide applications. However, resistance has reduced the efficacy of most registered products and the ability of the insect to become resistant to synthetic insecticides.

SLW has demonstrated an outstanding capacity for developing a high level of resistance to many insecticides. Public concern over the hazards associated with the synthetic insecticides, and the rapid resistance development within insect populations against the synthetic products has evoked interest on alternative pest control strategies. Plant origin products, as alternative to conventional insecticides, have potential benefit in IPM programs.

The objective of this study was to study the efficacy of various cypress extracts and fractions against silverleaf whitefly life stages under laboratory conditions.

Materials and Methods

Two separate experiments were conducted, the first to assess the direct mortality against whitefly adults, and the second to evaluate the effect on egg hatchability and nymphs mortality.

In both experiments, a total of eighteen extracts or fractions with an untreated control and a standard insecticide (Confidor® 200SC, applied at the label rate - diluted 0.25 mL/L in water (0.05 g a.i./L)) were evaluated against SLW adult, egg and nymph stages. For each treatment, 0.5 g of cypress extract or fraction was dissolved in 5.0 mL ethanol, and then 2 mL of the dissolved extract was mixed with 0.2 mL of non-ionic surfactant (Wetter 600®) and 98 mL of distilled water to produce a 0.2% spray solution. The surfactant (0.2 mL) and 2.0 mL ethanol were added to distilled water which served as the control.

Experiment 1. Adult mortality

Tomato leaves (5–7 leaflets on each leaf) were selected from SLW infestation free plants. Approximately 5 ml of the spray solution was sprayed on to each leaf with a hand-held mister and allowed to dry for 3–4 hours. The leaf stalk was immersed in a glass vial that filled with distilled water. Around 20–30 whitefly adults were released to each leaf, which was covered with a plastic cup (Photo 1).

All the treatments were arranged in a randomised block design with four replicates in a controlled temperature cabinet, where the temperatures were programmed at four different set-points (20, 23, 25 and 28°C) and with 50–60% relative humidity and photoperiod of 12:12 LD. Adult mortality was recorded at 2 and 5 days after treatment (DAT).

Photo 1: Plastic cup setup used for the adult mortality experiment

Experiment 2. Egg hatchability and nymph mortality

Tomato seedlings (variety Guardian) were grown in insect proof cages to avoid SLW infestation. When the seedlings were in the 4-week old stage, they were trimmed, leaving only four young leaves, and planted individually in 12 cm plastic pots.

The potted plants were placed in insect cages and SLW adults were released for them to lay eggs on the leaves. After 4 days, adults were removed and eggs were counted on four leaflets of each plant (pre-treatment egg counts).

Approximately 15 ml of the spray solution was sprayed on to each treatment replicate with a hand-held mister and allowed to dry for 3 hours. The treated plants were covered with a cylindrical film cage. All 20 treatments were arranged in a randomised block design with four replicates in a controlled temperature cabinet where the temperatures were programmed at four different set-points (20, 23, 25 and 27 °C) and with 50–60% relative humidity and photoperiod of 12:12 LD.

The four leaves from each cage were assessed under microscope to record hatched, unhatched eggs and nymph stages on 13 and 20 DAT.

Results and Discussion

Effect on adult mortality

The results of the cypress extracts against adult mortality are shown in Table 1 and Figure 1. The results showed that some cypress extracts do have direct mortality effect on SLW adults.

Among the cypress extracts tested A, A12, A13, A14, B, B13, B14, B15 and C12 showed high level of adult mortality, (90–100%) within 2 days (Table 1), indicating that these extracts may have quick knock-down effect on adults. This level of adult mortality was comparable to the standard insecticide, Confidor® 200 SC.

All other fractions showed significant ($p<0.5$) level of adult mortality compared with the untreated control however the level of mortality was well below the standard treatment in some cases.

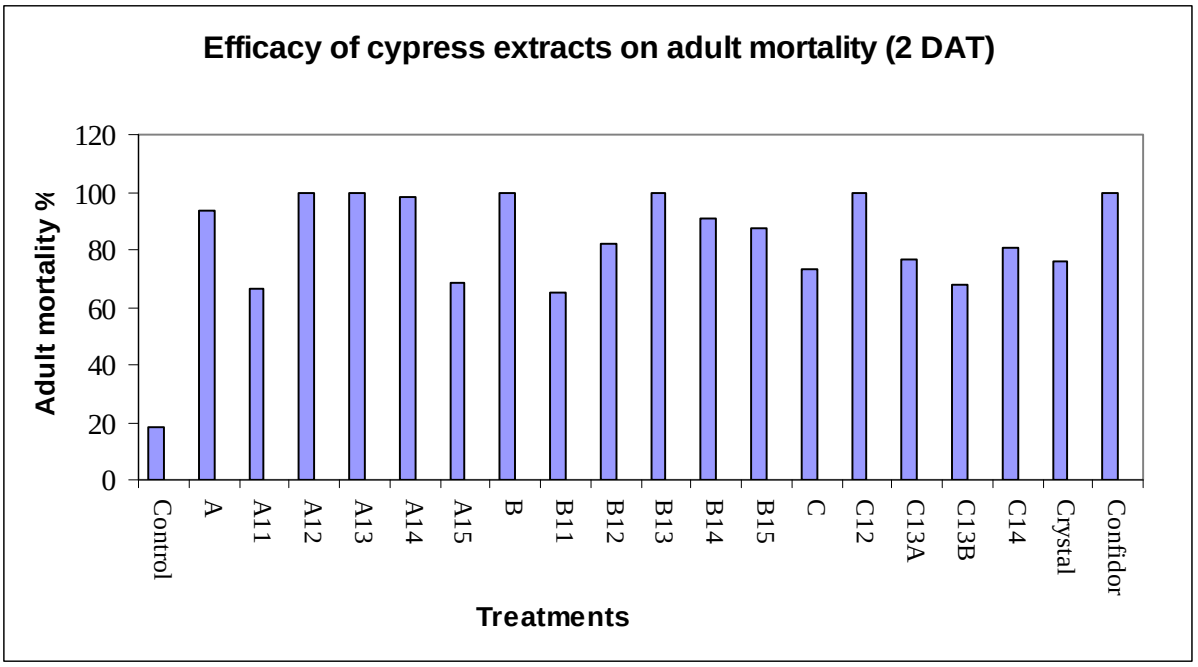


Figure 1**.** Efficacy of cypress extracts on adult silverleaf whitefly adults.

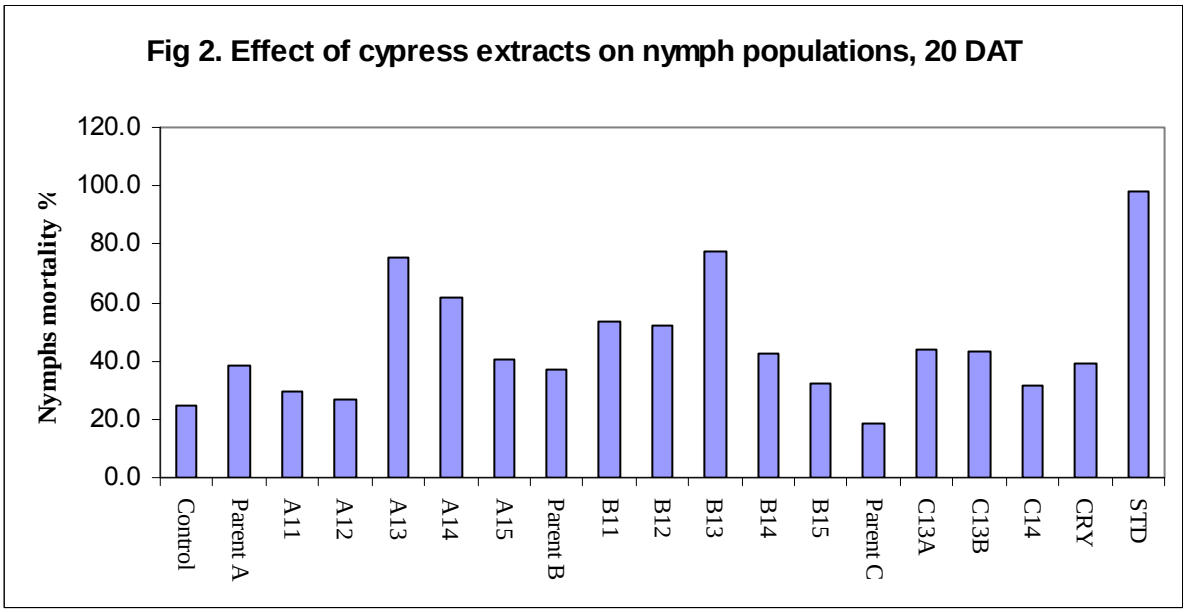
Effect on egg hatchability

The mean number of hatched eggs and percent egg hatchability are shown in Table 2. These results suggest no direct effect on egg mortality or hatchability. All treatments (including industry standard) showed low levels of egg mortality and the reductions were not significantly different ($p>0.05$) when compared with untreated control. It was noticed that most eggs were hatched and reached the first instars nymph stage at first assessment date (13 DAT).

Effect on nymph mortality

The treatments **A13, B13** and Confidor® 200 SC (industry standard) had significantly ($p>0.05$) fewer nymphs than other treatments (Figure 3) at 13 and 20 DAT. The nymph densities in the A13 and B13 were 50 to 60% lower than in the untreated control, which was comparable to the industry standard. This may be due to the high level of mortality or desiccation that occurred at the “crawler” stages. In the B14 treatment, the second assessment (20 DAT) has not been completed because of leaf burn that caused plant death in two replicates.

All other treatments did not significantly reduce the nymph numbers compared with the untreated control (Table 3). The nymph survival rate was higher in these treatments that this may have increased their establishment and development.



Conclusions

- The two fractions, A13 and B13 caused high level adult and nymph mortalities under the controlled condition. Also both fractions were reasonably safe on foliage and did not cause significant burn on leaves (phytotoxic scores below 1.25).
- Over all results shows that the extracts A13 and B13 have insecticidal properties, which can cause adult mortality and interfere in nymph populations. However, further studies may be required to understand the "mode of action" of these two fractions.
- In the experiment-1, SLW adults appeared to be more susceptible to most of extracts and fractions than the egg and nymph stages.
- The fraction C12 caused severe leaf burn on tomato plants; therefore this fraction was not evaluated in this study.

Table 1 Efficacy of cypress extracts against silverleaf whitefly adults

Treatments	Adult Mortality (%)	
	2 DAT	**5 DAT** (Cumulative)
Control	18.0 a	45.0 a
A (Full Extract)	93.5 b	100.0 c
A11 (Fraction)	66.7 b	73.7 b
A12	100.0 c	100.0 c
A13	100.0 c	100.0 c
A14	98.0 c	100.0 c
A15	68.2 b	72.9 b
B (Full Extract)	100.0 c	100.0 c
B11 (Fraction)	65.1 b	70.9 b
B12	82.3 bc	89.1 c
B13	100.0 c	100.0 c
B14	90.6 b	94.7 c
B15	87.3 bc	93.4 c
C (Full Extract)	73.3 b	81.9 b
C12	100.0 c	100.0 c
C13A	76.6 b	89.3 c
C13B	67.9 b	75.5 b
C 14	80.9 bc	88.3 c

Crystals	75.8 b	84.2 c
Confidor® 200 SC	100.0 c	100.0 c

Shaded cells in the same column indicate no significant difference from the standard Confidor® 200 SC
Means within column followed by the same letter did not differ significantly at the 5% level

Table 2: Efficacy of cypress extracts against egg hatchability, 13 DAT

Treatment	Pre treatment Mean eggs/ leaflet	Mean eggs hatched/ leaflet	Mean Egg hatchability (%)
Control	23.8	23.6	99.2 a
A (Full Extract)	17.8	17.5	98.3 a
A11 (Fraction)	17.8	17.5	98.3 a
A12	19.4	18.8	97.5 a
A13	29.7	29.4	99.5 a
A14	18.9	18.1	96.3 a
A15	15.0	14.3	96.5 a
B (Full Extract)	18.1	18.0	99.5 a
B11 (Fraction)	21.3	20.7	98.3 a
B12	14.2	13.8	97.0 a
B13	13.8	13.2	98.1 a
B14	22.2	21.0	96.7 a
B15	23.5	23.5	100.0a
C (Full Extract)	18.4	16.3	98.0 a
C12	NA*	NA*	NA*
C13A	21.3	20.9	98.3 a
C13B	23.7	23.5	99.4 a
C 14	23.6	23.4	99.4 a
Crystals	21.0	20.2	97.6 a
Confidor® 200 SC	21.8	17.9	86.2 a

Shaded cells in the same column indicate no significant difference from the standard Confidor® 200 SC
Means within column followed by the same letter did not differ significantly at the 5% level
NA* = C12 fraction caused severe burn on leaves so that full data were not collected.

Table 3: Efficacy of cypress extracts against nymphs mortality (13 and 20 DAT)

Treatment	Nymphs mortality % (13 DAT)	Nymphs mortality % (20 DAT)	Phytotoxic score #
Control	6.6 a	24.7 a	0.75
A (Full Extract)	29.0 a	38.1 a	1.00
A11 (Fraction)	11.1 a	29.7 a	1.50
A12	15.9 a	26.5 a	1.00
A13	53.8 b	75.6 bc	1.25
A14	39.9 a	61.5 b	1.00
A15	16.5 a	40.7 a	1.25
B (Full Extract)	9.6 a	37.2 a	1.50
B11 (Fraction)	28.3 a	53.3 a	1.75
B12	47.8 b	52.1 a	0.75
B13	69.7 bc	77.4 bc	1.00
B14	54.9 b	NA	2.75
B15	20.2 a	32.0 a	1.25
C (Full Extract)	13.0 a	18.3 a	2.25
C12*	NA	NA	4.00*
C13A	23.3 a	43.8 a	0.75
C13B	10.9 a	43.0 a	0.75
C 14	17.4 a	31.5 a	1.50

Crystals	4.0 a	39.3 a	1.00
Confidor® 200 SC (Standard)	94.6 c	97.9 C	0.25

Shaded cells in the same column indicate no significant difference from the standard confidor at the 5% level.
Means within column followed by the same letter did not differ significantly at the 5% level
DAT = days after treatment
NA* = C12 fraction caused severe burn on leaves therefore nymph data not available for the treatment.
# Phytotoxicity score: 1 = marginal or slight tip burn; 2 = around 5 – 20% leaf area burned;
3 = around 20 – 40 leaf area burned; 4 = over 40% of leaf area burned.

Appendix H: Testing Cypress extracts for efficacy against adult cattle ticks *(Boophilus microplus) – first screening*

Researcher Lex Turner, Principal Veterinary Officer, Animal Science, DPI&F

Background

A large proportion of the Queensland dairy industry is situated in the area infested with cattle ticks. Currently chemicals are used to routinely treat these ticks. There are major problems with using chemicals and alternatives need to be developed. The ticks develop resistance to these chemicals and the newer chemicals are usually more expensive. Consumers are also demanding lower chemical residue levels in dairy products and less chemical contamination of the environment. This trial will investigate the potential of Cypress extracts as tick control agents.

Materials and Methods

Cypress extracts

Supplied extracts consisted of parent extracts A, B and C and fractions A1, A2, A3, A4 and B1, B2, B3, B4 and C1, C2, C3, C4, C5, C6. numbered as follows:

Extract	Number
'A'	1
A1	2
A2	3
A3	4
A4	5
'B'	6
B1	7
B2	8
B3	9
B4	10
'C'	11
C1	12
C2	13
C3	14
C4	15
C5	16
C6	17
Blank control	18

Preparation of test dilutions and controls

The concentration of the extracts in the bioassay plates was 5%. This was prepared by dissolving 0.5 g extract in 4.5 mL (2% Tween 80 in ethanol) and adding 5 mL water. Ticks in control wells were treated with the diluting solution.

Determination of extract efficacy

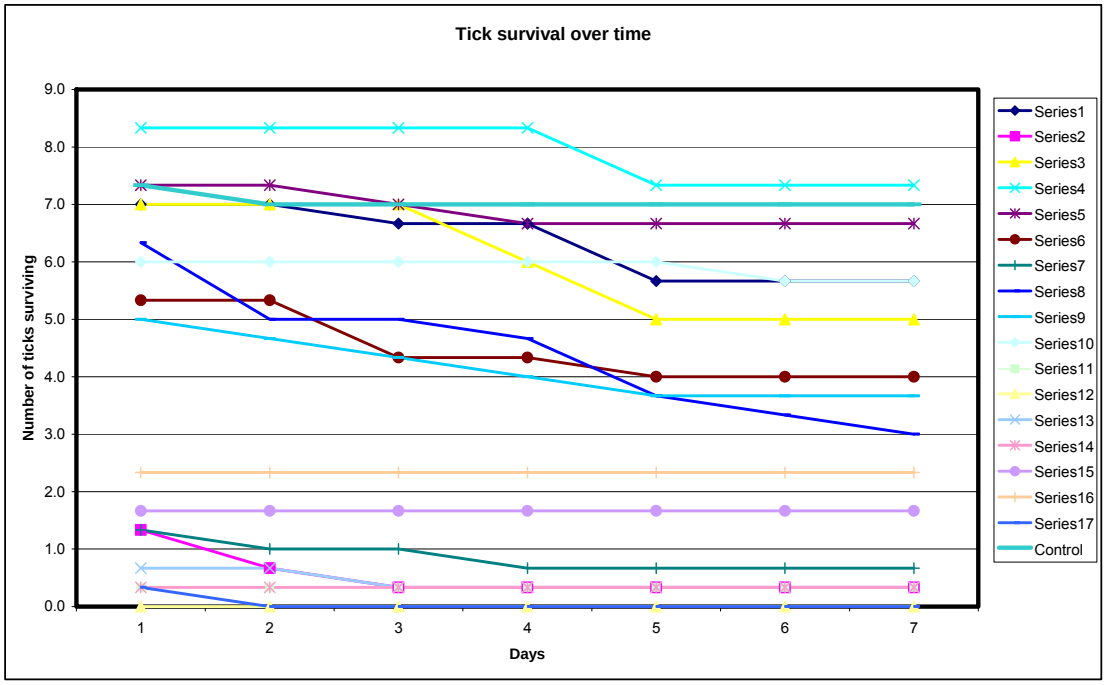
The ticks used were a non-resistant field strain obtained from the Department of Primary Industries and Fisheries at Yeerongpilly. Adult cattle ticks (*Boophilus microplus*) were immersed in the extracts and placed in individual wells in 24 well plates. Ten ticks on a plate were treated and this was replicated three times for each extract. The ticks were incubated at 27^0C with minimal humidity. The ticks were then checked daily for tick death and results were compared to control ticks (Table 1).

Results and Discussion

Table 1. Average number of ticks surviving (out of 10) recorded daily for 7 days

	Days						
Group	1	2	3	4	5	6	7
1	7.0	7.0	6.7	6.7	5.7	5.7	5.7
2	1.3	0.7	0.3	0.3	0.3	0.3	0.3
3	7.0	7.0	7.0	6.0	5.0	5.0	5.0
4	8.3	8.3	8.3	8.3	7.3	7.3	7.3
5	7.3	7.3	7.0	6.7	6.7	6.7	6.7
6	5.3	5.3	4.3	4.3	4.0	4.0	4.0
7	1.3	1.0	1.0	0.7	0.7	0.7	0.7
8	6.3	5.0	5.0	4.7	3.7	3.3	3.0
9	5.0	4.7	4.3	4.0	3.7	3.7	3.7
10	6.0	6.0	6.0	6.0	6.0	5.7	5.7
11	0.3	0.3	0.3	0.3	0.3	0.3	0.3
12	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13	0.7	0.7	0.3	0.3	0.3	0.3	0.3
14	0.3	0.3	0.3	0.3	0.3	0.3	0.3
15	1.7	1.7	1.7	1.7	1.7	1.7	1.7
16	2.3	2.3	2.3	2.3	2.3	2.3	2.3
17	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Control	7.3	7.0	7.0	7.0	7.0	7.0	7.0

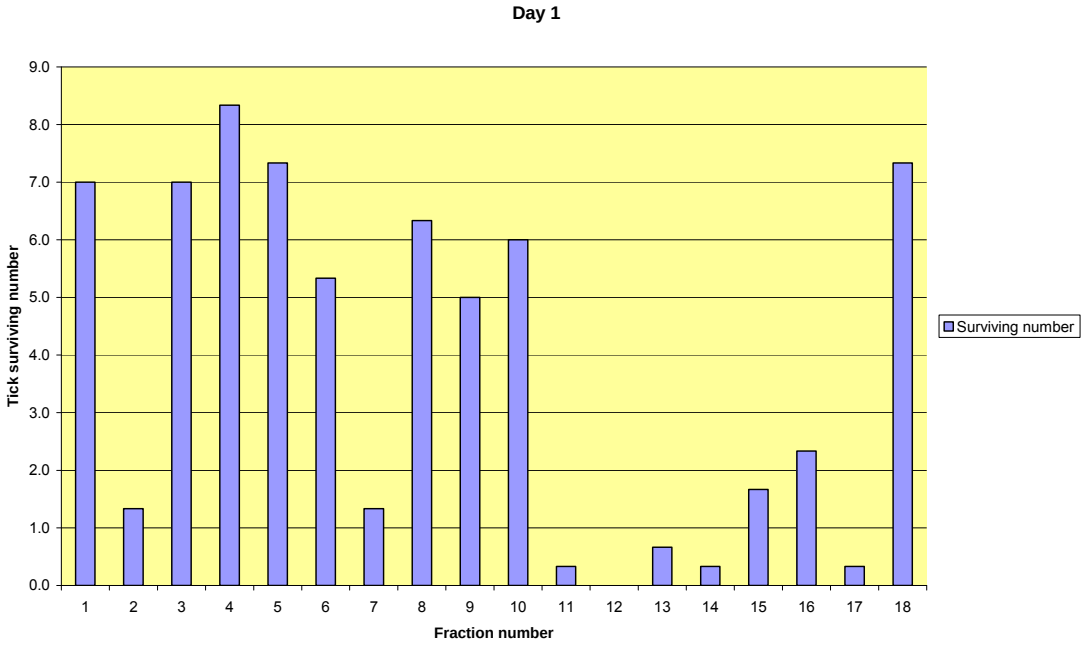
Figure 1. Daily tick survival when extracts were applied to adult ticks.



Tick deaths were most noticeable the day after treatment and most extracts did not kill many more over the next few days (Table 1 and Figure 1). It seems that if the ticks could survive the initial exposure then they had a reasonable chance of surviving for a few days. The A1 fraction was the most effective from the A extract, the B1 fraction was the most effective from the B extract and the C1 fraction was the most effective from the C extract. The fractions from the C extract were much more effective than those from the A or B extracts.

Day 1 was specifically graphed (Figure 2) as it represents the most effective period of action of the extracts and fractions. Several fractions (A1, B1, C, C1, C2, C3, C4, C5, C6) displayed reasonable day one efficacy.

Figure 2. Average tick survival after Day 1



Discussion

Further testing of the extracts would target those that were effective at 5% and should include testing at a lower concentration. The extracts not effective at 5% would seem to have limited potential against cattle ticks. Five percent would normally be a high percent for an oil addition to a cattle tick control solution. At this percentage, cost of oil inclusion would become important to the formulation of a commercial product.

These tests were conducted under the controlled conditions in the laboratory. These results may not be replicated on cattle, as the effect on ticks may be different on cattle compared to the laboratory because of all the external influences acting on cattle on the farms. External factors, wind, licking and sunshine, would be more likely to hinder the action of the extract rather than enhance the action.

When developing a tick control agent it is also important to note that these studies were not conducted on cattle and high concentrations of some of these extracts may have an impact on cattle. Some essential oils, including a pine (*Pinus sylvestris*) essential oil, have been tested for genotoxicity (Lazutka *et al.* 2001). Genotoxic potential was measured as the number of chromosomal aberrations caused by the oil. Pine essential oils were found to exhibit a dose-dependent genotoxicity and were also found to be mutagenic in the insect bioassay.

Other anti-tick uses of these extracts could be developed if they had a repellent effect. These extracts were not tested for any repellent effect in these trials but other studies have shown potential tick repellent effects of other plant essential oils. The essential oil of the shrub *Cleome hirta* was repellent to ticks in trials in Kenya (Ndungu 1999). It was considered useful for further investigation. Other work in Kenya (Lwande *et al*. 1999) looked at the repellency of the essential oil of a reported anti-tick pasture shrub *Gynandropsis gynandra*. It was found to be repellent but this repellency may have been due to toxic chemicals in the plant.

These products are not registered for tick control on cattle in this form.

References

Lazutka, J.R., Mierauskiene, J., Slapsyte, G., Dedonyte, V. (2001). Genotoxicity of dill (*Anethum graveolens* L), peppermint (*Mentha x piperita* L) and pine (*Pinus sylvestris* L) essential oils in human lymphocytes and *Drosophila melanogaster*. Food and Chemical Toxicology 39:485–492.

Lwande, W., Ndakala, A., Hassanali, A., Moreka, L., Nyandat, E., Ndungu, M., Amiani, H., Gitu, P.M., Malonza, M.M., Punyua, D.K. (1999). *Gynandropsis gynandra* essential oil and its constituents as tick (*Rhipicephalus appendiculatus*) repellents. Phytochemistry 50:401–405.

Ndungu, M.W. (1999). *Cleome hirta* essential oil as a livestock tick (*Rhipicephalus appendiculatus*) and maize weevil (*Sitophilus zeamais*) repellent. *Fitoterapia* 70:514–516.

Recommendation

That evaluation of some of the cypress extracts for efficacy against adult cattle ticks be continued.

Appendix I: Testing Cypress extracts for efficacy against adult cattle ticks *(Boophilus microplus)*; second screening test

Researcher Lex Turner, Principal Veterinary Officer, Animal Science, DPI&F

Background

The cattle tick (*Boophilus microplus*) is a major ectoparasite of cattle in tropical and subtropical areas of the world including Australia. It can cause serious debilitation such as weight loss in infested animals as well as significant economic and production losses due to worry and the blood meal ingested by engorging ticks. In addition, the cattle tick is a vector for a number of blood-borne pathogens that cause tick fever in cattle. This disease can cause serious morbidity and mortality in susceptible animals.

The control of the cattle tick in the field is primarily by use of chemicals. Unfortunately the cattle tick has demonstrated an excellent capacity to develop resistance to groups of chemicals within a relatively short period of time. The newer chemicals being developed are usually more expensive. Consumers are also demanding lower chemical residue levels in dairy products and less chemical contamination of the environment. This trial will investigate the potential of cypress extracts as tick control agents to replace some chemical usage.

Materials and Methods

Cypress extracts

Supplied specimens consisted of crude extracts A, B and C and fractions A11, A12, A13, A14, A15 and B11, B12, B13, B14, B15 and C12, C13A, C13B, C14, and C5. These were labelled:

Extract or fraction	Labelled
'A'	1
'B'	2
'C'	3
A11	4
A12	5
A13	6
A14	7
A15	8
B11	9
B12	10
B13	11
B14	12
B15	13
C12	14
C13A	15
C13B	16
C14	17
C5	18
Blank Control	UT
Treated Control	TC

Preparation of test dilutions and controls

The concentration of the extracts in the bioassay plates was 2%. This was prepared by dissolving 0.5 g extract in 4.5 mL ethanol and adding 8 mL water to 2 mL of the solution. Ticks in control wells were either treated with the diluting solution or had no treatment.

Determination of extract efficacy

The ticks used were sourced from the Department of Primary Industries and Fisheries' Animal Research Station at Yeerongpilly, Brisbane. Adult cattle ticks (*Boophilus microplus*) were immersed briefly in the extracts before being placed in individual wells in 24 well plates. Ten ticks on a plate were treated;

replicated three times for each extract. The ticks were incubated at 27^{0}C with minimal humidity, and checked daily for tick death and results were compared to control ticks.

Results and Discussion

Table 1. Average number of ticks still alive (out of 10) recorded daily for 4 days

Label	Extract or fraction	Day 1	Day 2	Day 3	Day 4
1	'A'	9.5	9.0	9.0	9.0
2	'B'	9.0	7.0	7.0	5.3
3	'C'	7.3	4.3	3.7	2.3
4	A11	9.7	9.7	9.7	9.3
5	A12	9.7	9.7	9.7	9.7
6	A13	10.0	10.0	10.0	10.0
7	A14	10.0	10.0	10.0	10.0
8	A15	10.0	9.7	9.7	9.7
9	B11	9.7	9.3	9.3	9.3
10	B12	8.3	6.0	6.3	4.0
11	B13	10.0	9.7	9.3	9.3
12	B14	9.7	9.3	9.3	9.0
13	B15	10.0	10.0	10.0	10.0
14	C12	10.0	8.7	8.0	7.0
15	C13A	9.7	9.3	9.3	9.3
16	C13B	1.0	0.3	0.0	0.0
17	C14	4.3	3.0	2.0	0.7
18	C5	10.0	10.0	10.0	10.0
TC	Untreated Control	9.3	8.7	8.7	8.7
UT	Treated Control	9.7	9.7	9.7	9.7

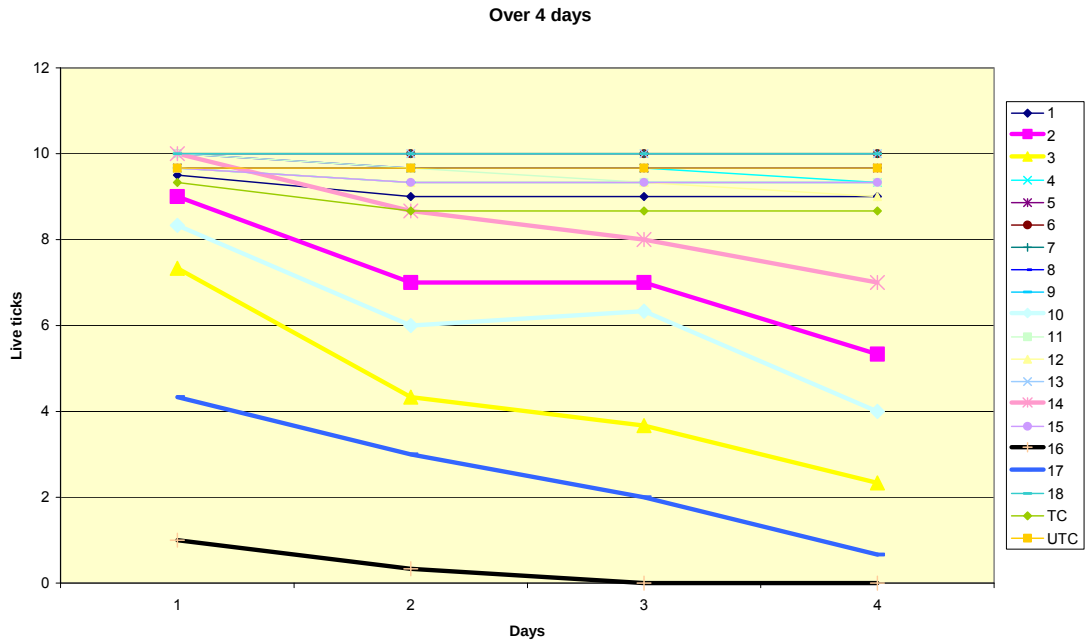


Figure 1.. The average number of adult ticks surviving over 4 days after extract application

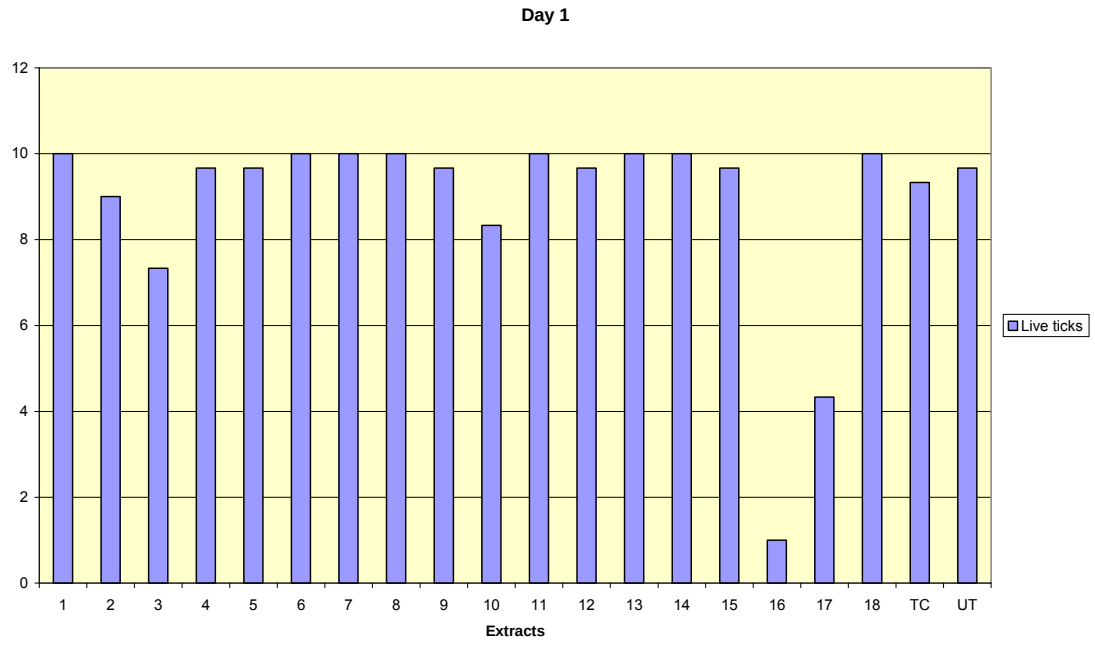


Figure 2. The average number of ticks surviving on day 1.

Day 1 was specifically graphed as it demonstrates the extracts with a rapid action. Only two extracts (C13B and C14) displayed reasonable day one efficacy. A number of other extracts continued to kill ticks over the four days and displayed reasonable efficacy by day 4.

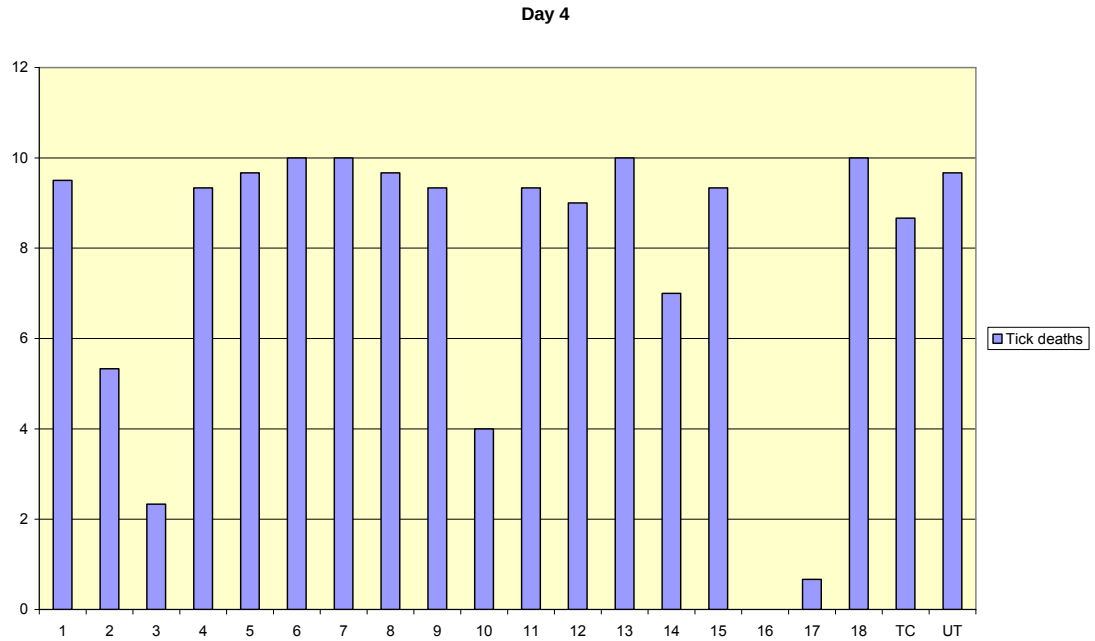


Figure 3. Average tick survival after 4 days.

Extracts showing promise at this time include C, B12, C13B and C14. All ticks that had died by day 4 had failed to lay eggs. This is important for efficacy of tick treatments as these ticks do not then complete their life cycle.

Extract	Extract or fraction	Ticks
1	'A'	9.5
2	'B'	5.0
3	'C'	0.0
4	A11	9.3
5	A12	9.0
6	A13	10.0
7	A14	10.0
8	A15	9.7
9	B11	9.3
10	B12	0.3
11	B13	7.7
12	B14	9.0
13	B15	10.0
14	C12	4.0
15	C13A	8.7
16	C13B	0.0
17	C14	0.3
18	C5	9.3
TC	Untreated Control	8.7
UT	Treated Control	9.3

Table 2. The average number of ticks laying eggs by day 4.

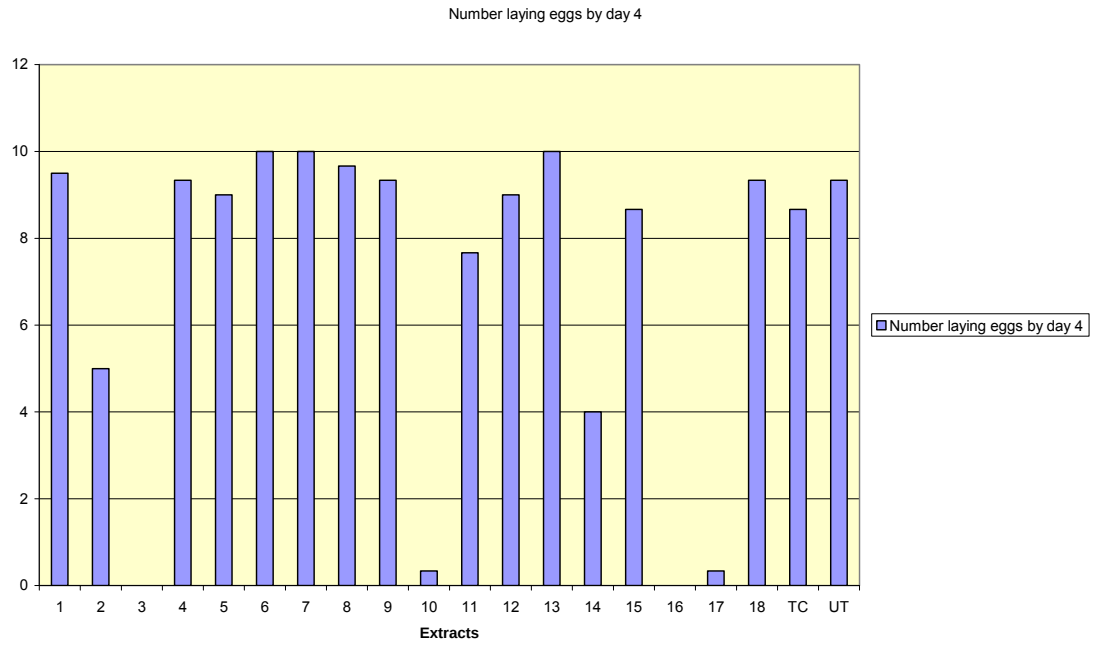


Figure 4. The average number of ticks laying eggs by day 4.

The egg laying results correlate well with the number of ticks surviving in each treatment group. The most promising extracts at reducing tick egg laying include C, B12, C13B and C14.

Discussion

Those extracts that were effective at 2% could be tested at a lower concentration. The extracts not effective at 2% may have limited potential against cattle ticks depending on price of the extract and effect of the oil on the cattle themselves. All extracts showing reasonable efficacy may be useful as an additive in other tick control solutions.

The extracts C, B12, C13B and C14 may be useful as tick control agents without the inclusion of other agents. If 2% of the extract is too expensive or too irritant to the cattle, the concentration could be reduced if the oil could be mixed with other tick control agents.

These tests were conducted under the controlled conditions in the laboratory. These results may not be replicated on cattle, as the effect on ticks may be different on cattle compared to the laboratory because of all the external influences acting on cattle on the farms. External factors, wind, licking and sunshine, would be more likely to hinder the action of the extract rather than enhance the action.

When developing a tick control agent it is also important to note that these studies were not conducted on cattle and some of these extracts may have an impact on cattle. Some essential oils, including a pine (*Pinus sylvestris*) essential oil, have been tested for genotoxicity (3). Genotoxic potential was measured as the number of chromosomal aberrations caused by the oil. Pine essential oils were found to exhibit a dose-dependent genotoxicity and were also found to be mutagenic in the insect bioassay.

Other anti-tick uses of these extracts could be developed if they had a repellent effect. These extracts were not tested for any repellent effect in these trials but other studies have shown potential tick repellent effects of other plant essential oils. The essential oil of the shrub *Cleome hirta* was repellent to ticks in trials in Kenya (1). It was considered useful for further investigation. Other work in Kenya (2) looked at the repellency of the essential oil of a reported anti-tick pasture shrub *Gynandropsis gynandra*. It was found to be repellent but this repellency may have been due to toxic chemicals in the plant.

These products are undergoing testing in a laboratory and are not registered for tick control on cattle in this form.

References

Lazutka, J.R., Mierauskiene, J., Slapsyte, G., Dedonyte, V. (2001). Genotoxicity of dill (*Anethum graveolens* L), peppermint (*Mentha x piperita* L) and pine (*Pinus sylvestris* L) essential oils in human lymphocytes and *Drosophila melanogaster*. Food and Chemical Toxicology 39:485–492.

Lwande, W., Ndakala, A., Hassanali, A., Moreka, L., Nyandat, E., Ndungu, M., Amiani, H., Gitu, P.M., Malonza, M.M., Punyua, D.K. (1999). *Gynandropsis gynandra* essential oil and its constituents as tick (*Rhipicephalus appendiculatus*) repellents. Phytochemistry 50:401–405.

Ndungu, M.W. (1999). *Cleome hirta* essential oil as a livestock tick (*Rhipicephalus appendiculatus*) and maize weevil (*Sitophilus zeamais*) repellent. *Fitoterapia* 70:514–516.

Recommendation

Four of the specimens tested (crude extract ‘C’ and fractions B12, C13B and C14) showed reasonable efficacy against adult ticks at the 2% level, killing many of the ticks within 4 days and preventing most of the egg laying. These may be useful products at the 2% level or at a lower level if added to other tick control agents. Cost and effects on cattle need to be considered. Further testing and refining of these extracts is recommended for their potential for controlling ticks.

Appendix J: The larvicidal activity of cypress extracts against *Haemonchus contortus* of sheep and goats

Researcher Maxine Lyndal-Murphy, Snr Parasitologist, Animal Science, ARI Yeerongpilly

Background

Haemonchus contortus is a pathogenic gastrointestinal nematode of small ruminants pastured in the tropical and sub tropical regions of Australia. The repeated use of chemical anthelmintics to control this parasite has inadvertently selected nematodes resistant to the treatments leaving many producers with limited chemical options. The estimated cost to the industry in Australia if resistance continues to escalate is estimated to be $200 million by the year 2010.

Materials and Methods

Cypress extracts

Supplied extracts consisted of parent extracts A, B and C and fractions A1, A2, A3, A4 and B1, B2, B3, B4 and C1, C2, C3, C4, C5, C6.

Preparation of test dilutions and controls

Extracts and fractions were prepared according to the supplied method to produce a 0.2% concentration. This concentration was then serially diluted twofold in 1% Tween 80 to achieve 0.1% and 0.05% dilutions. Final concentrations of the extracts in the bioassay plates were 0.1%, 0.05% and 0.025%. Negative control wells contained water or Tween 80 (final concentration of 0.5 %) and the positive control wells (third stage only) contained levamisole discriminating dose (0.5% final concentration).

Determination of in vitro larvicidal activity

First stage larval motility assay:

Nematode eggs were recovered from faeces of a donor sheep (Le Jambre 1976) carrying a natural infection of *Haemonchus contortus* and hatched overnight at 26°C. About 100 first stage larvae in 150 µl of water were distributed in 48 well flat-bottomed plates and exposed to an equal volume of each dilution at 26ºC for 24 hours. There were 4 replicates of each dilution. Motility was assessed under X60 magnification and scored according to O'Grady and Kotze (2004) with modifications to suit the test type.

Third stage larval motility assay:

Faeces collected from the same donor sheep were cultured at 26ºC for 7 days and recovered according to Lyndal-Murphy (1993). About 100 third stage larvae in 150µl of water were exposed to an equal volume of each dilution in 48 well flat-bottomed plates at 26ºC for 24 hours. There were 4 replicates of each dilution. Motility was assessed under x60 magnification and scored according to O'Grady and Kotze (2004) with modifications to suit the test type.

Results and Discussion

The results of the in vitro determination showed that cypress extracts do have larvicidal activity against *H contortus*.

The first stage larvae were observed to be more susceptible to the toxic effects of the extracts and fractions than the third stage. This finding is consistent with observations of Lyndal-Murphy M (DPI/Mediherb Report – 2002).

First stage motility test

- Overall, the C group was observed to be more active than either A or B with B being more active than A.
- A comparison of the parent extracts A, B and C indicated that A was as active as C with B the most active at the 0.025% dilution.
- The data for the A group showed contradictory toxicity; the parent extract A was more potent than any of its fractions that individually had little effect at 0.05% dilution. This observation is unexpected and needs to be retested.
- The fractions C2, C4, and C6 were observed to be the most potent with toxic affects (motility score of 0) down to 0.025%. B2 and to a lesser extent B3 also exerted toxic effects (motility scores of 1 and 1.5 respectively indicating strong paralysis with spastic movements in some) at 0.025% dilution.

Data are summarized in Table 1. Negative controls scored a motility of 4 indicating no inhibition of motility.

Third stage motility test

- The activity of all extracts and fractions against the third stage larvae resulted in a mild paralysis of only a small proportion of the exposed larvae and was a consistent finding across all extracts and fractions. Negative controls scored a motility of 4 indicating no effect on motility while the positive control scored a motility of 0 indicating all third stage larvae were severely paralysed.

The sub-fractions C1, C2 and C5 precipitated out when mixed with the water containing the larvae. The needle-like crystals were obvious on microscopic examination. This may have adversely influenced the activity of the C1 and C5 fractions.

Conclusions

The results of this investigation show that the first stage larvae of *H contortus* are more susceptible to the toxic effects of the cypress extracts than the third stage larvae and these findings are consistent with those observed for data on other essential oils derived from selected native Australian trees (Lyndal-Murphy M). The level of activity of the C series is also consistent with that of other essential oils used in these types of bioassays and the earlier work of CSIRO (McKern and Parnell -1964).

Recommendation

That further testing of the cypress extracts for novel larvicidal activity against *Haemonchus contortus* be continued.

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Le Jambre, L.F. (1976). Egg Hatch as an *in vitro* assay of thiabendazole resistance in nematodes. Veterinary Parasitology. 2**:**385–391.

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McKern, H.H.G., Parnell, I.W., 1964, The Larvicidal Effect of Various chemical Coumpounds and Plant Products on the Free-Living Stages of Haemonchus cotortus Rud. (Nematodes). Journal of Helminthology. 38:223–244.

O'Grady, J. and Kotze, A.C. (2004). H*aemonchus contortus*: in vitro drug screening assays with the adult life stage. Experimental Parasitology 106:164–172.

Table 1. *Motility (average) of first-stage larvae of *H contortus* in different dilutions (%) of cypress extracts in vitro. Motility scoring after O'Grady and Kotze (2004).

	Dilutions %				Dilutions %				Dilutions %		
Crude extract & fractions	0.1	0.05	0.025	Crude extract & fractions	0.1	0.05	0.025	Crude extract & fractions	0.1	0.05	0.025
'A'	0	0	3	**'B'**	0	0	2	**'C'**	0	0	3
A1	1	2.5	4	**B1**	1	1.5	2	**C1**	0	2	3
A2	0	2	4	**B2**	0	0	**1**	**C2**	**0**	**0**	**0**
A3	2	3	4	**B3**	0	0	1.5	**C3**	0	0	3
A4	3	4	4	**B4**	0	1	2	**C4**	**0**	**0**	**0**
								C5	0	0	3
								C6	**0**	**0**	**0**

*Motility: 0, dead or severely paralysed; 1, slight twitching: 2, few with normal movements; 3, half with normal movements; 4, normal movements in most similar to those at the start of the study.

Extracts with most potency.

Appendix K: The invitro larvicidal activity of cypress extracts against *Haemonchus contortus* of sheep and goats; second screening test

Researcher Maxine Lyndal-Murphy, Snr Parasitologist, Animal Science, ARI Yeerongpilly

Materials and methods

Cypress extracts

Crude extracts 'A', 'B' and 'C' and fractions C12, C13A, C13B, C14 and C15 were tested.

Preparation of test dilutions and controls

Extracts and fractions were prepared according to the supplied method with the following modifications: the 10% solutions were further mixed with Tween 80 and then water was added to produce a 0.1% concentration containing 0.3% Tween80. Twofold serial dilutions from 0.1% to 0.001% achieved test concentrations of 0.05% to 0.0005%. Extracts were assessed against a positive (essential oil, non industry standard) and negative controls (water and Tween 80 final concentration of 1.5%).

Determination of in vitro larvicidal activity

First stage larval motility assays were performed as previously reported (Screening of cypress extractives against pests & diseases – 1, Kennedy *et al*. 2006). A modification of the motility scoring system allowed statistical analysis to be conducted: only normal motility was scored as an indicator of efficacy - Box 1.

Results

All cypress extracts achieved larval mortality at some of the concentrations tested but the positive essential oil control treatment (non industry standard) was significantly superior ($P<0.001$). The results demonstrated that the activity of the parent extracts A, B and C were not significantly different from each other; each produced 100% mortality at 0.05% dilution, comparable with the efficacies of the same parent extracts in the preceding screening test. C12 demonstrated the most potent activity with 100% kill at 0.006% dilution followed by C13A at 0.0125% dilution with C13B, C14 and C15 producing a 100% mortality at 0.025% dilution, compared to the 100% mortality at 0.0012% dilution achieved by the control essential oil. Negative controls had no effect on the larvae. Exponential curves relating normal sinusoidal motility (expressed as a motility score, Graphs, Box 1) to concentration were fitted to the data (see Graphs, Table 1 and Table 2). Extract C14 appeared incompletely dissolved as evidenced by the needle-like structures observed in the test solutions.

Discussion and Conclusion

These extracts displayed activity more potent than many other essential oil extracts reported in the literature as achieving anti-parasitic activity against *H. contortus* in vitro (Pessoa *et al.* 2002 and Lyndal-Murphy M, personal communication). The extracts C12 and C13A were the most potent of the actives tested with C12 more potent than C13A. C12 achieved an activity that should be further tested in vitro against adult *Haemonchus contortus* harvested from sheep.

References

Kennedy, M.J., Halfpapp, K., Fay, H., Hargreaves, J.R., Astridge, D., Subramaniam, S. Sivasubramaniam, V., Lyndal-Murphy, M. and Turner, L. (2005). Screening of cypress extractives against pests & diseases – 1. Appendix J: The larvicidal activity of cypress extracts against Haemonchus contortus of sheep and goats Milestone 1 of FWPRDC Project PN04.2006: Commercial products from bio-active extractives in cypress milling residues.

Pessoa, L.M., Morais, S.M., Bevilaqua, C.M.L., Luciano, J.H.S., (2002). Anthelmintic activity of essential oil of *Ocimum gratissimum* Linn. and eugenol against *Haemonchus contortus*. Veterinary Parasitology 109, 59-63

Graphs

Effect of cypress extracts A, B and C on the motility of first stage Haemonchus contortus larvae in vitro

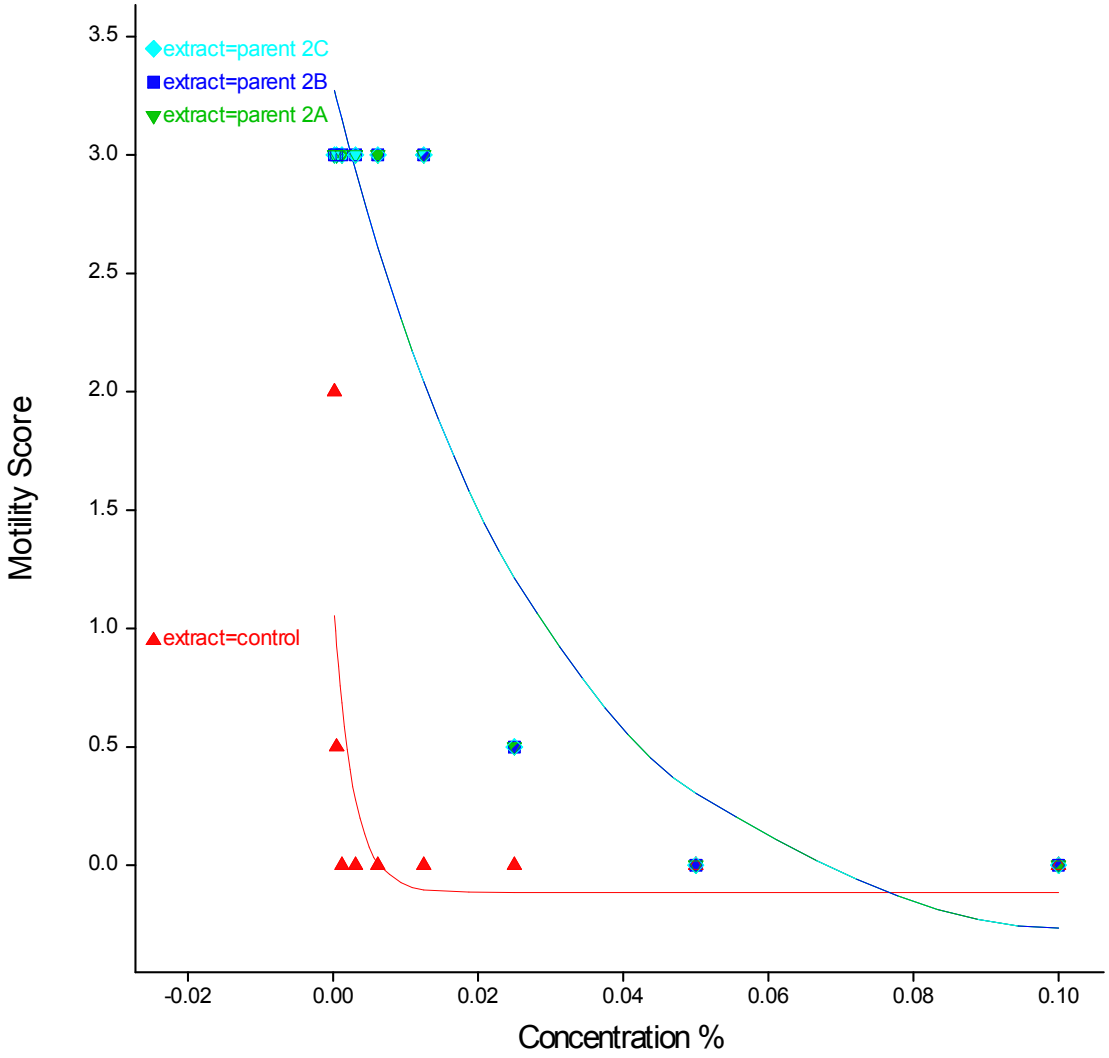

Table 1. The fitted curves modelled the crude extract data well (R^2=85.4%, P<0.001).

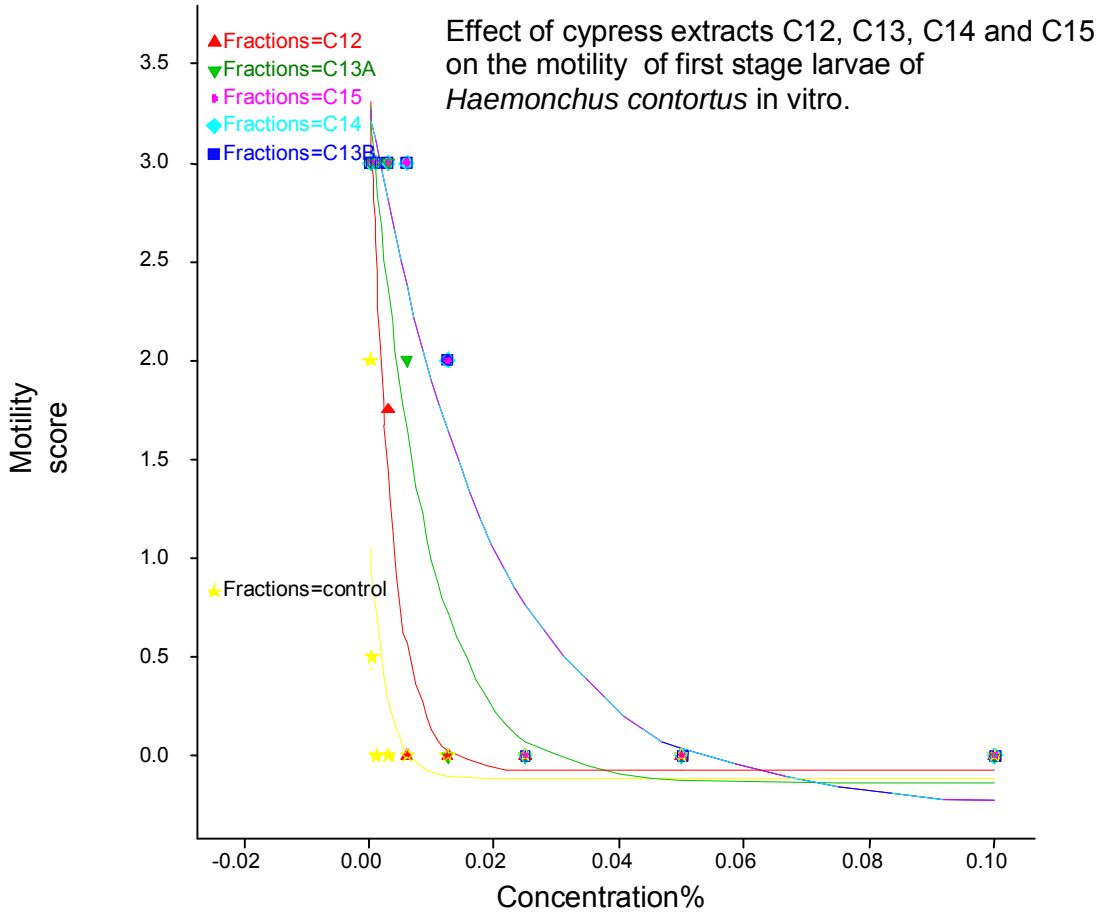

Table 2. The fitted curves modelled the fraction data well (R^2=90%, P<0.001).

Box 1

Motility scores
0= larvae dead
1= at least one larvae with normal sinusoidal motility
2= about half of the larvae with normal sinusoidal motility
3= most larvae with normal sinusoidal motility the same as at the start of the test

Appendix L: Third series of screening of cypress extracts against silverleaf whitefly (*Bemisia tabaci)* on tomato

Researchers Siva Subramaniam, and Verni Sivasubramaniam, DPI&F, Bowen, Queensland

Introduction

This third series of screening experiments against silverleaf whitefly (SLW) was undertaken for the same overall purposes outlined in the introductions to previous reports (Appendix F and G), and to further explore the activity of fractions of crude extract 'C' and some of its major groups of components and individual components.

Materials and methods

These experiments assessed the direct mortality of selected chemical extractive specimens against whitefly adults and nymphs.

A total of fourteen fractions with an untreated control and a standard insecticide (Confidor® 200SC, applied at the label rate - diluted 0.25 mL/L in water (50 mg a.i./L)) were evaluated against SLW adult and nymph stages. For each treatment, 0.5 g of cypress extract or fraction was dissolved in 5.0 ml ethanol, and then 2 mL of the dissolved extract was mixed with 0.2 mL of non-ionic surfactant (Wetter 600®) and 98 mL of distilled water to produce 0.2% spray solution. The surfactant (0.2 mL) and 2.0 mL ethanol were added to distilled water which served as the control.

Experiment 1

Tomato plants were sprayed with 0.2% of fractions C21 to C30 from the third fractionation experiment, or with controls, and allowed to air dry for an hour. A known number of SLW adults were introduced to each treatment and covered with cylindrical cages and kept at 25°C. After 2 days surviving adults were counted. Again after 5 days surviving adults were counted and the adults were removed. The plants were again incubated for a week to measure effect on egg numbers and hatchability. After 13 days - leaves were removed from each plant and the hatched nymphs counted.

Experiment 2

Tomato plants were infested with SLW and kept for 10 days for eggs and nymphs to develop. The infested leaves were then removed from the adults and the number of eggs and nymphs counted. The leaves were dipped in 0.2% aqueous suspensions of C25, C27, C28, C29 or control, and incubated for 4 days. After 4 days, mortality of nymphs was assessed. Fractions C25 and C27 produced leaf burn at 0.2%, rendering the C27 data unusable. The experiment was subsequently repeated for fractions C25 and C27 at lower concentration (0.1%).

Experiment 3

Tomato plants were sprayed with 0.1% of extracts C31 to C37, subfractions and mixtures of components from the third fractionation experiment, or with controls, and allowed to air dry for an hour. A known number of SLW adults were introduced to each treatment and covered with cylindrical cages and kept at 25°C. After 2 days surviving adults were counted. Again after 5 days surviving adults were counted and the adults were removed. The plants were again incubated for three weeks to measure effect on egg numbers and hatchability. After 24 days - leaves were removed from each plant and the hatched nymphs counted.

Results and Discussion

Experiment 1

Mortality of SLW adults is given in Figure 1, and the effect of reduced oviposition and egg hatchability on the number of nymphs produced is given in Figure 2.

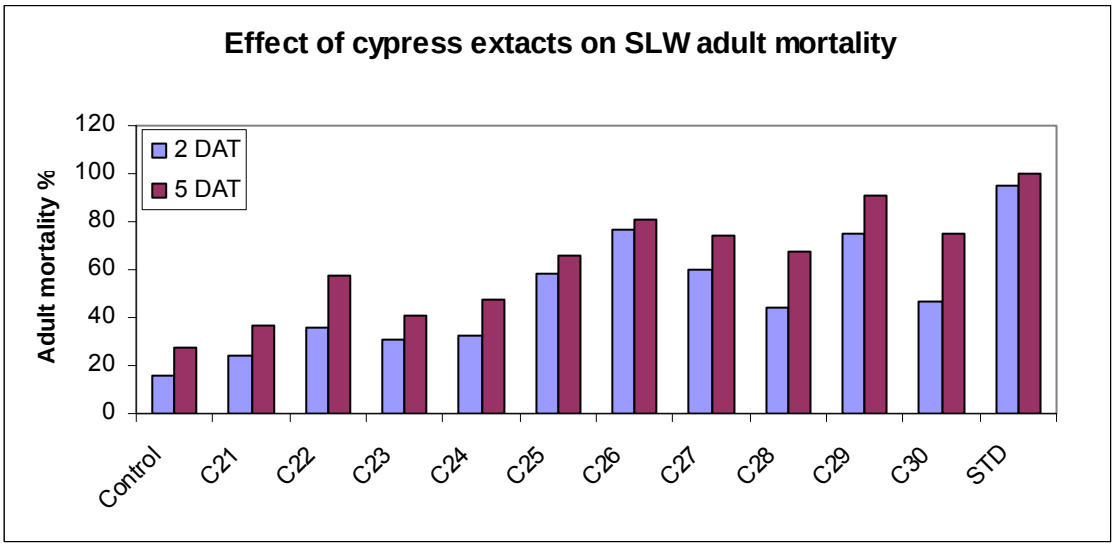

Figure 1: Effect of treatments on mortality of SLW adults

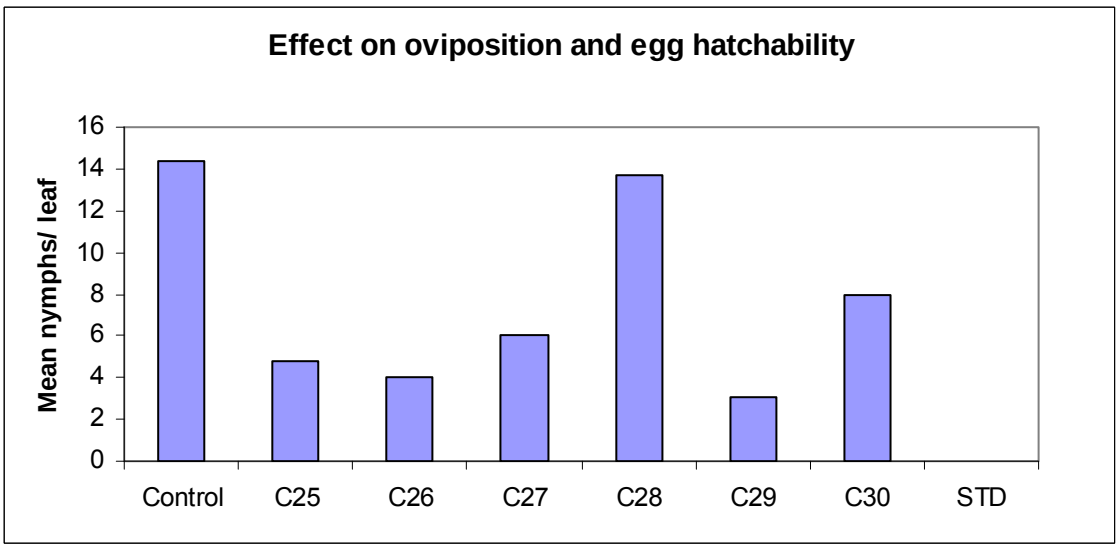

Figure 2: Effect of pre-infection treatments on next generation of SLW nymphs

In treatments C25 to C30, adult mortality ranged from 65 to 91%. C29 was superior to others and similar to the imidacloprid control. This was further supported with low level of nymph numbers on the leaves in C29, C26 and C25. The adult mortality levels in C21 to 24 were low - 25 to 58%

Experiment 2

Effect of fractions applied to pre-established eggs and nymphs is given in Figure 3.

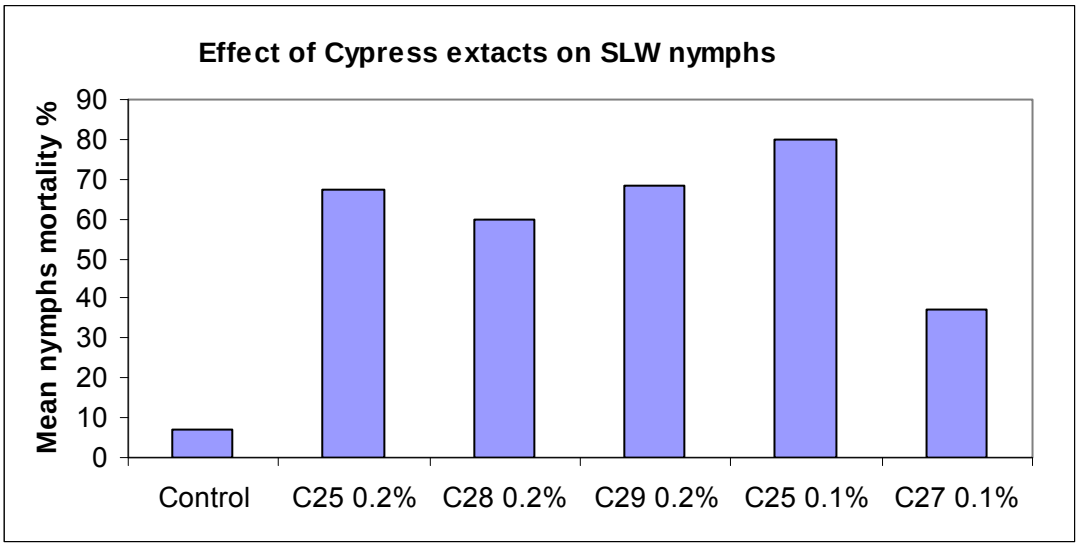

Figure 3: Effect of post-infection treatments on next generation of SLW nymphs

C27 burned leaves of all three replicates at 0.2 % concentration, preventing data collection. One of the three replicates of C25 at 0.2% was not burned – the C25 outcome in Figure 3 was generated from this one replicate. One of the three replicates of C29 inadvertently dried out during the experiment, two replicate data were collected and are presented here. C25 at 0.1 % gave excellent control at up to 79 % mortality; C27 at 0.1% was inferior.

Among these fractions, C29 performed better in controlling adults as well as nymphs. C25 gave better control for nymphs but less for adults. C26 gave better control for adults, but it was not tested for direct mortality on nymphs.

Experiment 3

Mortality of SLW adults is given in Figure 4, and the effect of reduced oviposition and egg hatchability on the number of nymphs produced is given in Figure 5.

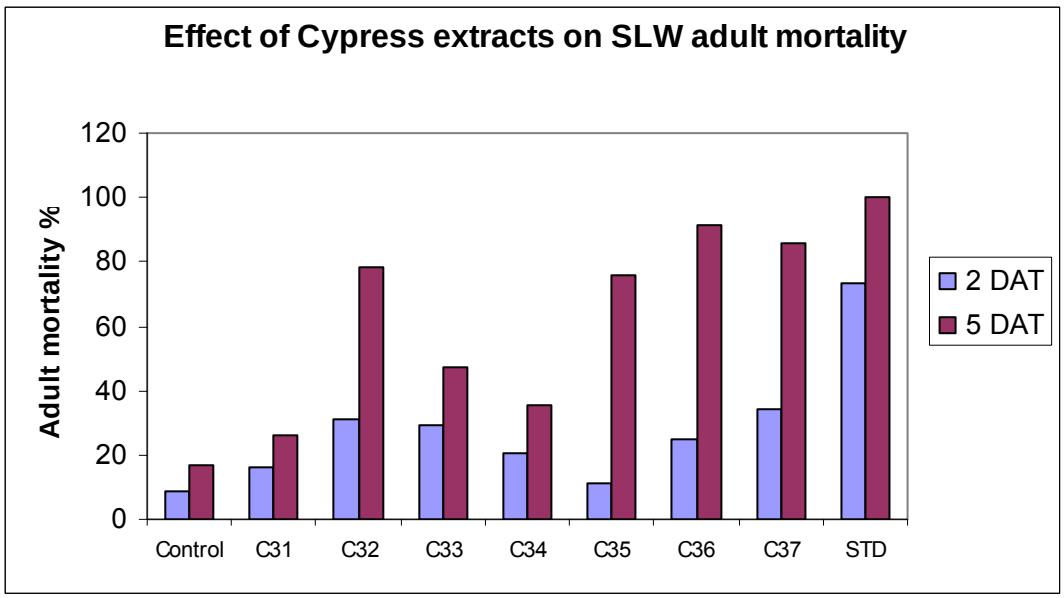

Figure 4: Effect of treatments on mortality of SLW adults

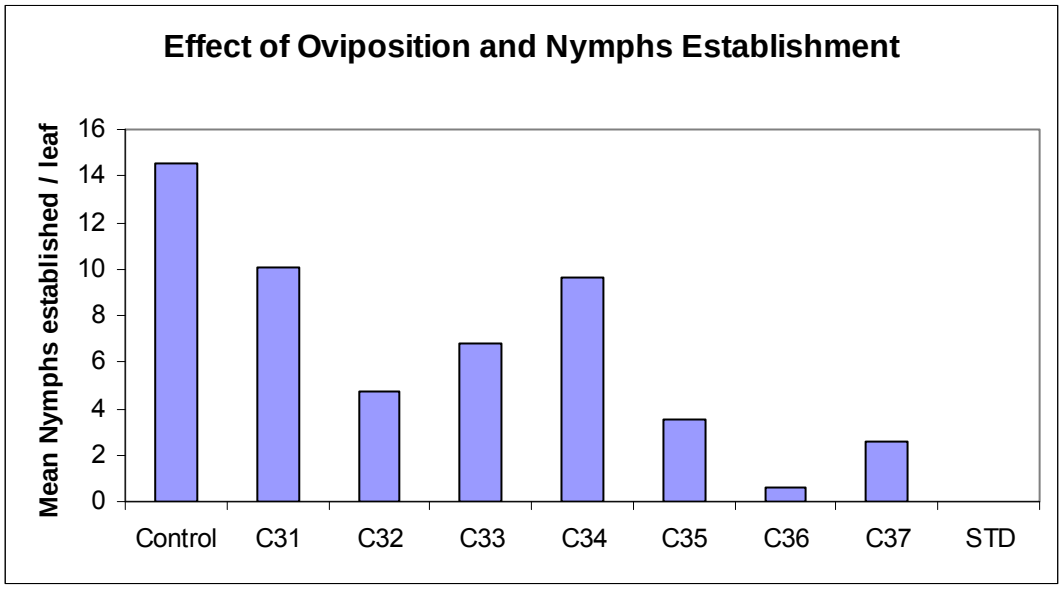

Figure 5: Effect of pre-infection treatments of sub-fractions on next generation of SLW nymphs

Among the 7 sub-fractions . C32, C35, C36 and C37 gave significant levels of adult mortality, ranging between 76 to 92%. C36 performed better than others – the mean adult mortality level was 92%, similar to the imidacloprid control. Adult mortality levels for C31, C33 & C34 were not useful – range 26 to 46%. C36 controlled nymph development very well, limiting to 0.6 nymphs/ leaf. In C37, and C35, the nymph numbers were around 2.6 and 3.5 nymphs/ leaf compared with 14.5 nymphs/ leaf in untreated control. At the 0.1 % concentration, none of the extracts caused leaf burn on tomato leaves.

Conclusions

Screening data were generated to support an assessment of the relative contributions of various components of crude extract ‘C’ to control of silverleaf whitefly. The assessment is provided in the main report.

Appendix M: Efficacy of cypress extracts and mixtures with paraffinic oils against silverleaf whitefly (*Bemisia tabaci)* on eggplant

Researchers Brendan Nolan, Mary Firrell and Ron Hermann, Horticulture & Forestry Science, Gatton, Qld 4343

Introduction

Silverleaf whitefly (*Bemisia tabaci*), b-biotype, is a serious pest of a wide range of horticultural crops. Due to its short lifecycle, rapid population growth and resistance to many insecticides, new methods of control are constantly being sought.

Initial screening of cypress extracts was undertaken at the DPI&F Research Station, Gatton where a number of potentially effective compounds against silverleaf whitefly (SLW) were identified. We report here results from efficacy testing of various extract formulations against adult SLW

Materials and Methods

The experiment was conducted at the Department of Primary Industries and Fisheries Research Station, Gatton, Queensland between October 2007 and December 2007. In this experiment, crude extract 'C' at 3 rates, alone or in combination with Sacoa Biopest® oil (a total of 6 chemical treatments), 4 controls and a standard treatment of Confidor® 200 SC (a.i.: imidacloprid) were assessed against SLW adults (Table 1).

Table 1: Treatments assessed during experiment.

Treatment	Treatment Code	10% crude 'C' Concentrate (mL)	Tween 80 (mL)	Ethanol (mL)	Biopest Oil (mL)
Crude Extract C @ 0.3%	41	30	4	0	0
Crude Extract C @ 0.3% + Biopest Oil @ 0.2%	42	30	4	0	2
Crude Extract C @ 0.2%	43	20	4	10	0
Crude Extract C @ 0.2% + Biopest Oil @ 0.2%	44	20	4	10	2
Crude Extract C @ 0.1%	45	10	4	20	0
Crude Extract C @ 0.1% + Biopest Oil @ 0.2%	46	10	4	20	2
Control A Dip: no cypress, no Biopest Oil	47	0	4	30	0
Control B Dip: Biopest Oil @ 0.2%, no cypress	48	0	0	0	2
Control D Dip: in water	49	0	0	0	0
Control C No dip	410				
Standard SLW control (0.25 mL Confidor & 0.1 mL Spreadwet® in 1L water)	411	0	0	0	0

Eggplant seedlings were purchased from a commercial nursery and checked under a microscope to ensure the plants were free of silverleaf whitefly adults and nymphs. The seedlings were transplanted into small plastic cells.

Each eggplant seedling was dipped into its corresponding treatment for 5 seconds. Plants were then allowed to dry for 3–4 hours and the plastic cup containing each plant was hot glued into the bottom of a 150 mm high, clear plastic drink cup and covered with a ventilated lid.

Twenty SLW adults were introduced to each cup. The treatments were arranged in a randomised block design with four replicates in an incubator set at 25°C and with a photoperiod of 12:12 LD. Adult survival and phytotoxicity scores was recorded at 1 and 4 days after treatment (DAT).

The effects of phytotoxicity caused to plants by different formulations were assessed by assigning criteria scores during plant assessment (Table 2).

Table 2. Criteria used to assess phytotoxicity effects

Phytotoxicity score	Phytotoxicity descriptor
1	marginal or slight tip burn
2	around 5–20% leaf area burned
3	around 20–40 leaf area burned
4	over 40–60% of leaf area burned
5	over 60–80% of leaf area burned
6	over 80–100% of leaf area burned

Results and Discussion

Adult mortality at 1 and 4 days after treatment (DAT) are displayed in Figures 1 and 2. All cypress treatments caused between 40 and 50% mortality of adult SLW at 1 DAT, which was similar to the treatment 411 (Confidor® 200 SC - standard industry insecticide). Treatments 41 to 46, and 48 were significantly different from the control treatment.

Mortality observed in the cypress treatments did not change greatly at 4 DAT with exception of treatment 42. This suggests the cypress extract does not have a very long residual effect. The higher mortality observed in treatment 42 at 4 DAT may be due to the high phytotoxicity (60%) caused by the cypress formulation (see results below). Death of the host plant may have in turn caused mortality of adult SLW due to lack of food.

Treatment 411 (Confidor® 200 SC - standard industry insecticide) also displayed a higher mortality at 4 DAT. Confidor® 200 SC is known to have a high residual capability which explains the continued mortality in this treatment.

The efficacy results suggest the cypress formulations tested can cause up to 50% mortality of SLW adults 24 hrs after application, which is similar to the results achieved by the insecticide Confidor® 200 SC.

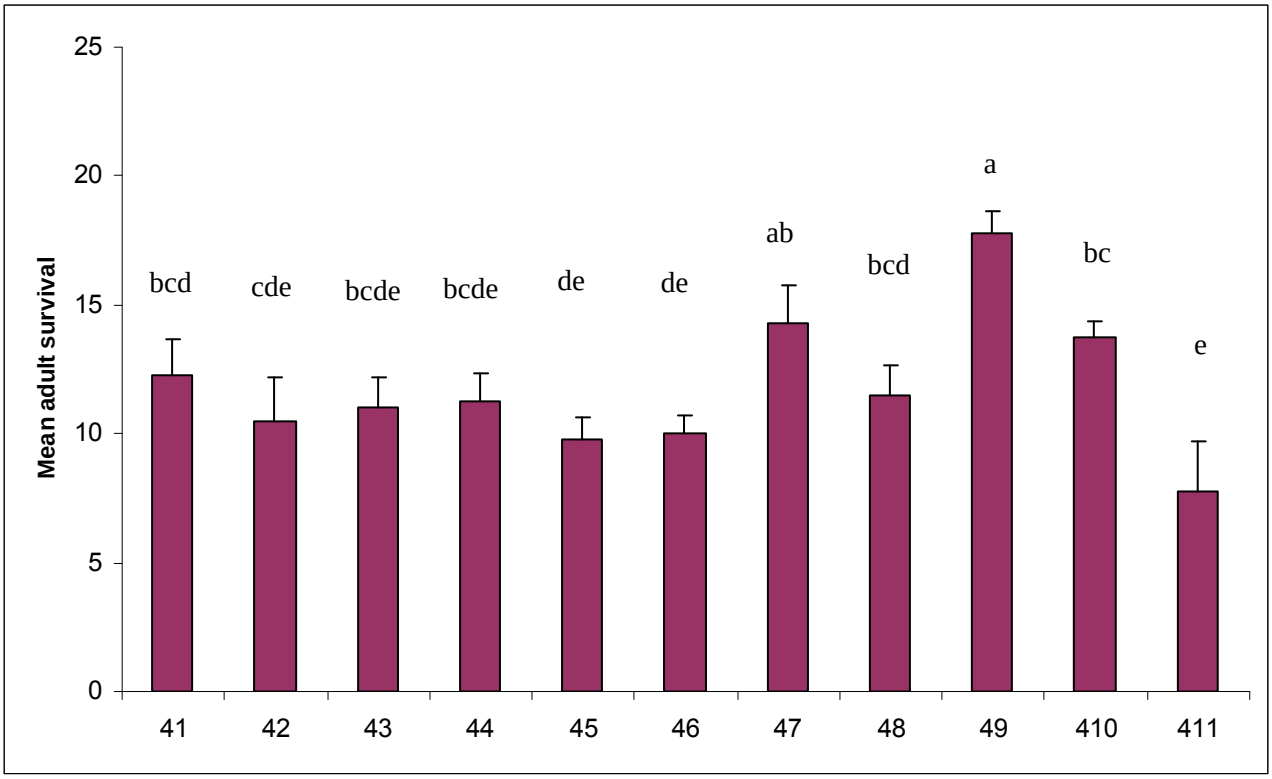

Figure1: Mean Survival of adult SLW 1 day after treatment. Treatments with the same letter are not significantly different.

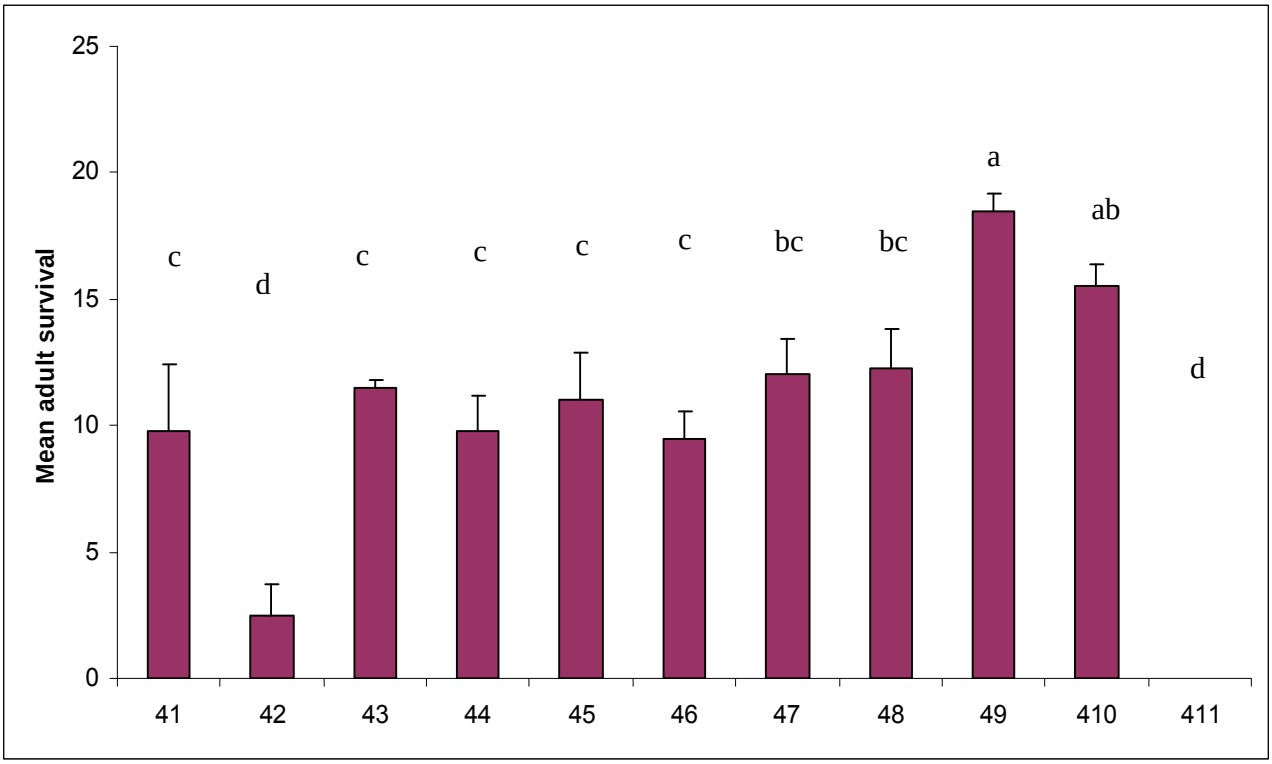

Figure 2 Mean Survival of adult SLW 4 days after treatment. Treatments with the same letter are not significantly different.

The mean phytotoxic effect on the plants from each treatment is shown in Figure 3 and 4. At 4 DAT, no phytotoxicity was observed in Treatments 45 to 411. Treatments 41, 43 and 44 received between 5 and 20% leaf burn at 4 DAT. While, treatment 42 (Crude Extract C @ 0.3% + Biopest Oil @ 0.2%) displayed phytotoxicity levels of 60%. Treatment 42's result may be due to the combination of cypress and Biopest oils. The majority of cypress formulations caused no leaf burn and would be suitable for use at these rates in commercial crops for the control of SLW.

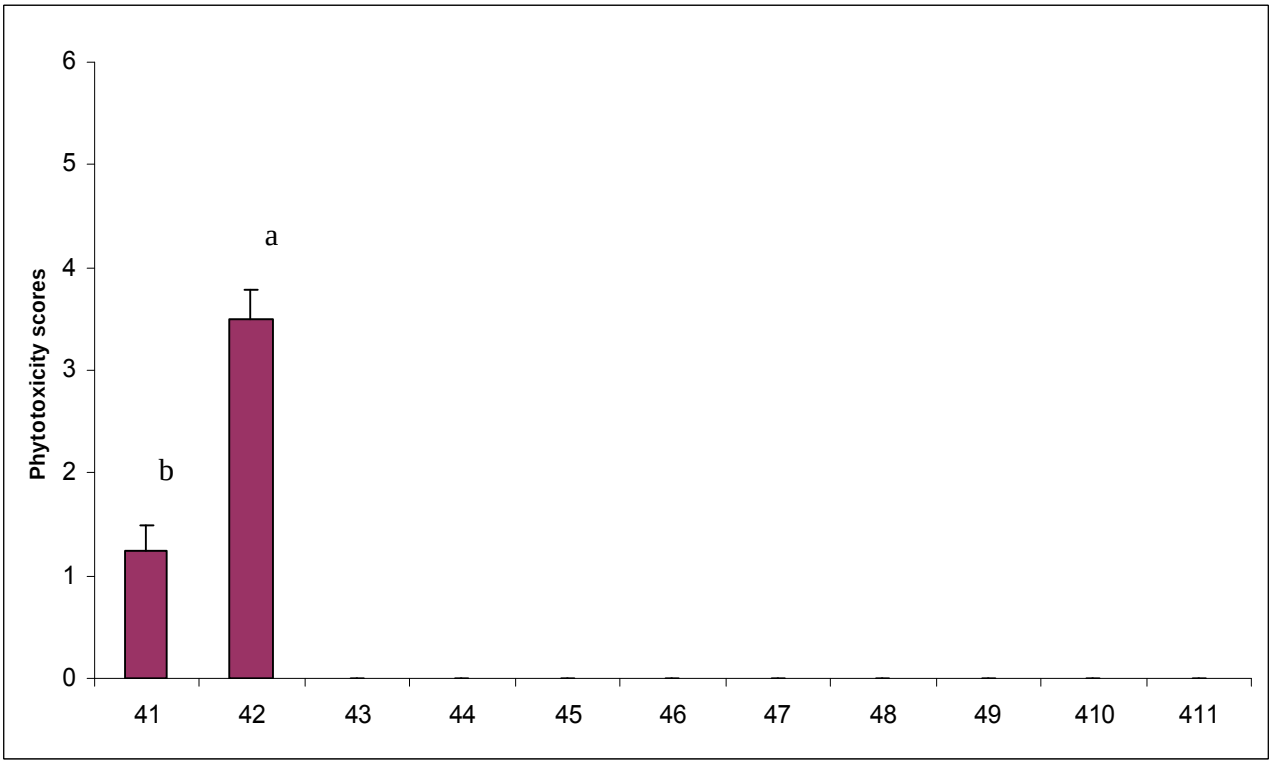

Figure 3: Mean Phytotoxicity Rating 1 Day after treatment. Treatments with the same letter are not significantly different.

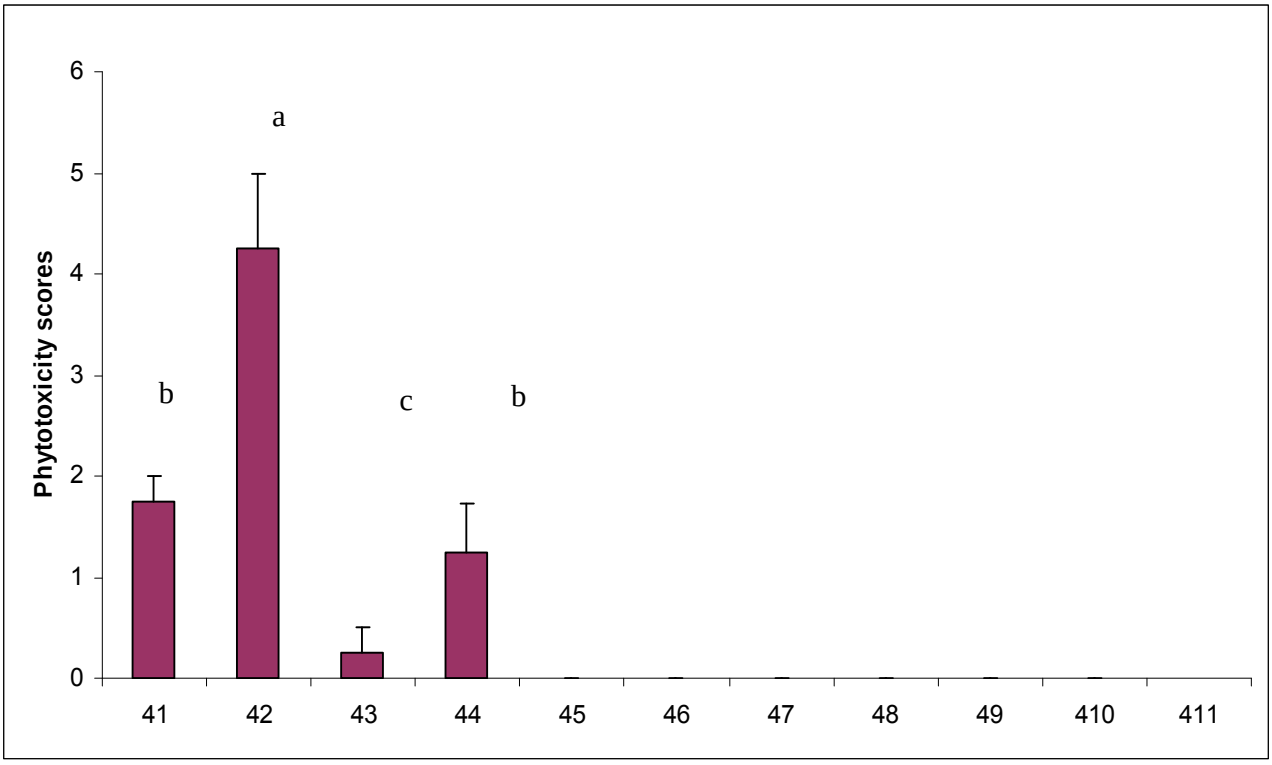

Figure 4: Mean Phytotoxicity Rating 4 days after treatment. Treatments with the same letter are not significantly different.

Acknowledgements

We thank David Schofield (Manager, Gatton Research Station, DPI&F) for supplying laboratory facilities.

Appendix N: Effect of cypress extracts on the parasitoid wasp *Eretmocerus hayati*

Researchers Brendan Nolan, Mary Firrell and Ron Hermann, Horticulture & Forestry Science, Gatton, Qld 4343

Introduction

Silverleaf whitefly (*Bemesia tabaci*) is a serious pest of a wide range of horticultural crops. Due to its short lifecycle, rapid population growth and resistance to many insecticides, new methods of control are constantly being sought.

Initial screening of cypress extracts were undertaken at Bowen, North Queensland, where a number of effective compounds were identified. Further testing was required to investigate whether these extracts would also affect *Eretmocerus hayati*, a parasitoid of silverleaf whitefly (SLW).

Materials and Methods

The experiment was conducted at the Queensland Department of Primary Industries and Fisheries Research Station, Gatton between October 2007 and December 2007.

In this experiment, 1 extract at 3 rates (a total of 3 chemical treatments), 6 controls and 1 standard treatment of Confidor® 200 SC were assessed against the aphelinid wasp *Eretmocerus hayati*. Each treatment was replicated 4 times.

Eggplant seedlings were purchased from a commercial nursery and checked under a microscope to ensure the plants were free of SLW eggs and nymphs. Forty individual leaves were selected and excised from Eggplant seedlings. The stem tip of each leaf was inserted into a wad of moist cotton wool then wrapped in plastic.

For each treatment, a concentrated solution of crude extract 'C' and other formulation components were mixed at the rates outlined in Table 1 and were diluted to 1000 mL in 1L beakers. Each plant was dipped for 5 seconds in the assigned treatment solution. Plants were allowed to air dry for 3 hours then they were placed into glass vials (25 mm x 100 mm). Into each vial, 20 adult parasitic wasps were placed. Parasitic wasps were sourced from a laboratory colony located at CSIRO's Long Pocket laboratories.

All 11 treatments were arranged in a randomised block design with four replicates in an incubator set at 25°C with a photoperiod of 14:10 LD. Adult survival was recorded at one Day After Treatment (DAT).

Table 1 Treatments assessed during experiment

Treatment	Treatment Code	10% crude 'C' concentrate (mL)	Tween 80 (mL)	Ethanol (mL)	Biopest Oil (mL)
Crude Extract C @ 0.3%	51	30	4	0	0
Crude Extract C @ 0.2%	52	20	4	10	0
Crude Extract C @ 0.1%	53	10	4	20	0
Biopest Oil @ 0.4%	54	0	0	0	4.0
Biopest Oil @ 0.2%	55	0	0	0	2.0
Crude Extract C @ 0.2% + Biopest Oil @ 0.2%	56	20	4	10	2.0
Control A Dip: no cypress, no Biopest Oil	57	0	4	30	0.0
Control B Dip: in water	58	0	0	0	0
Control C No dip	59				
Standard SLW control (0.25 mL Confidor & 0.1 mL Spreadwet® in 1L water)	510	0	0	0	0

Results and Discussion

Adult wasp mortality data recorded during the experiment are displayed in Figure 1. Because earlier experiments showed that crude extract 'C', when not supplemented with paraffinic oil, is not residual, additional monitoring greater than 1 day was not required.

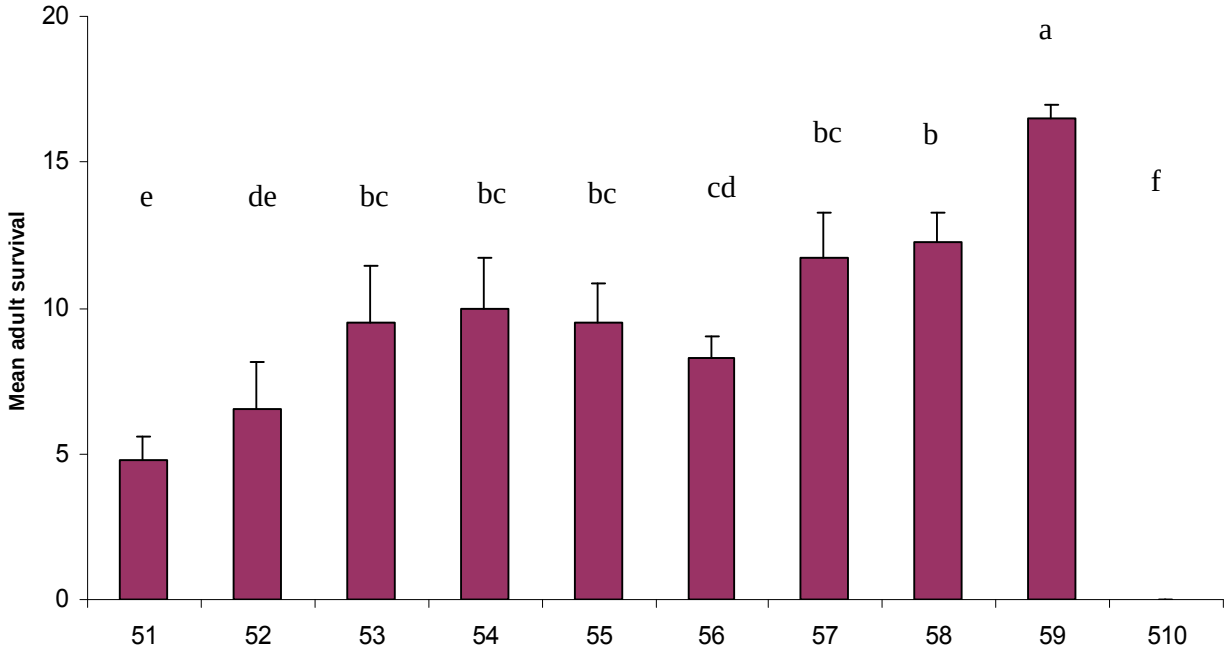

Figure 1. Mean mortality of adult parasitic wasps 1 day after treatment. Treatments with the same letter are not significantly different.

All treatments, including the controls received some mortality. The method used during this experiment is novel, and produced a small amount of mechanical error, approximately 15% and 40% mortality, as evident in the controls.

Considering this error all treatments, except 51, 52, 56 and 510, were not significantly different from the control treatments. Treatments 51, 52 and 56, which contained between 0.2% and 0.3% cypress extract, caused approximately 60 and 75% mortality. Treatment 510 (Confidor® 200 SC insecticide) caused 100% wasp mortality.

This result suggests that the cypress extract is disruptive to the parasitic wasps at rates of 0.1% or greater. However, it is not as toxic as the standard industry insecticide, Confidor® 200 SC. Because of the level of mechanical error, this experiment should be repeated to confirm results achieved.

Acknowledgements:

We thank David Schofield (Manager, Gatton Research Station, DPI&F) for supplying laboratory facilities and Andrew Hulthen (CSIRO, Long Pocket) for supplying the wasp *Eretmocerus hayati*.

Appendix O: Effect of cypress extracts on fungal disease organisms *Rosellinia necatrix* and *Alternaria mali*

Researcher Kathy Grice, Horticulture & Forestry Science, DPI&F, Mareeba, QLD 4880

Introduction

Rosellinia (or Dematophora, or white) root rot (*Roselinia nectarix*) and Alternaria blotch (*Alternaria mali*) are diseases of apple (*Malus* sp.). They were chosen as test organisms for evaluation of the disease control potential of cypress extracts, as they differ in their ecology, affecting root and foliage/fruit respectively, but both cause significant production losses annually and are not well controlled by currently registered products. Rosellinia root rot is known to cause serious tree losses. The disease is more prevalent in areas that have been cleared of vegetation susceptible to the organism and in replant situations. Alternaria blotch has become prevalent in the Granite Belt region of Queensland in recent years. The organism was initially recorded as a leaf spot and caused premature leaf drop (late in the season) up to 80% but did not appear to reduce production. More recently the organism has been observed to cause fruit damage particularly on the high value varieties (Royal Gala, Pink Lady and Braeburn), reducing production by 10% in some orchards.

Materials and Methods

The experiments were conducted at the Queensland Department of Primary Industries and Fisheries Laboratories in Mareeba. Each crude extract and each fraction from the first fractionation experiment was evaluated against each of the organisms at concentrations of 2000, 200, 20 and 2ppm in potato dextrose agar (PDA) media, with additional negative (blank check) controls.

Preparation of stock solutions

Stock solutions (2000ppm, 0.2%) were prepared from each parent and fraction of cypress extract material. Initial 10% solutions were prepared by dissolving 0.50mL of the test material in 5.0mL of acetone. 2000ppm solutions were prepared by adding 2.0mL of 10% solution to a sterile 100mL volumetric flask containing 0.2mL of Tween 80. This was mixed thoroughly before adding sterile distilled water to volume.

Preparation of media

2000ppm plates were prepared by adding 2.0mL aliquots of 10% stock solution to sterile 100mL volumetric flasks that contained 0.2mL of Tween 80. Melted and warm (50^{o}C) PDA was then added to volume. Each solution was agitated well and dispensed across eight petri dishes (90mm diameter).

The remaining plates, containing 200ppm, 20ppm and 2ppm, were prepared by taking 10mL of the required stock solution and adding 90mL of PDA, and proceeding as previously mentioned. Non-amended PDA was used as the check/control media. Plates were labelled with the product name, concentration and a cross to mark the centre of each dish then allowed to solidify and dry before use.

Organism preparation

5mm plugs cut from the edge of actively growing cultures of *R. necatrix* or *A. mali* were placed on 3-4 plates of PDA and incubated at approximately 26^{o} C for 2-3 days prior the start of each experiment.

Radial growth test

Plugs (5mm) were cut from the edge of actively growing 2-3 day old culture plates and inverted onto the marked centre of the PDA plates amended with the four concentrations of the cypress parents and fractions. The same process was used for non-amended control/check and was replicated four times. All petri dishes were sealed with parafilm to avoid drying out and contamination before placing in an incubator at approximately 26^{o}C for 3-4 days or until the organism had grown to the edge of the control/check plates. At 24hr intervals the diameter of the colonies was measured using digital callipers. In order to test all the products, five separate experiments were conducted between November 2004 and February 2005.

Results and Discussion

Radial growth data are given in Tables 1 and 2.

Table 1. Radial growth* of *Rosellina necatrix* after 3 days growing on PDA amended with cypress products.

Cypress product	Concentrations (ppm)				
	Nil	2	20	200	2000
Crude A	100	91.1	86.7	47.3	21.7
Fraction A1	100	74.7	76.8	41.7	21.8
Fraction A2	100	85.8	76.5	39.9	18.6
Fraction A3	100	92.9	57.2	56.9	23.8
Fraction A4	100	98.6	93.3	53.7	16.8
Crude B	100	91.7	83.4	39.9	15.9
Fraction B1	100	89.3	78.7	44.8	23.0
Fraction B2	100	85.9	63.3	32.5	14.7
Fraction B3	100	98.8	87.7	42.5	19.4
Fraction B4	100	90.0	85.4	41.6	6.9
Crude C	100	91.3	87.5	40.9	8.6
Fraction C1	100	81.5	87.4	56.2	35.5
Fraction C2	100	87.4	80.2	41.9	21.4
Fraction C3	100	97.5	93.4	42.6	11.9
Fraction C4	100	65.8	74.9	41.9	6.2
Fraction C5	100	99.8	102.8	82.1	47.9
Fraction C6	100	96.6	75.6	45.2	7.6

* The radial growth is expressed as a percentage of growth compared with the non-amended control.

Table 1. Radial growth* of *Alternaria mali* after 7 days growing on PDA amended with cypress products.

Cypress product	Concentrations (ppm)				
	Nil	2	20	200	2000
Crude A	100	90	86.1	56.7	20.7
Fraction A1	100	70.0	75.9	30.7	18.2
Fraction A2	100	83.9	69.1	30.6	19.3
Fraction A3	100	93.3	88.2	62.6	25.3
Fraction A4	100	90.5	91.5	64.5	24.8
Crude B	100	96.7	86.0	50.6	19.4
Fraction B1	100	98.5	77.7	26.2	19.3
Fraction B2	100	94.8	69.4	27.0	16.8
Fraction B3	100	96.0	82.2	48.9	22.6
Fraction B4	100	97.2	90.2	51.4	14.5
Crude C	100	94.5	87.8	39.6	10.5
Fraction C1	100	88.0	88.3	74.1	39.1
Fraction C2	100	85.6	63.2	22.4	17.2
Fraction C3	100	100.7	93	31	11.4
Fraction C4	100	88.5	88.0	32.7	9.2
Fraction C5	100	98.6	84.8	79.8	64.4
Fraction C6	100	94.5	87.6	42.5	10.4

* The radial growth is expressed as a percentage of growth compared to the non-amended control.

The data from each experiment were statistically analysed and the results show that there was no significant reduction in radial growth of either organism below 200ppm. At this concentration, 13 of the 17 products reduced radial growth of *R necatrix* by at least 50% compared with the untreated control. For fractions A1, C1, C4 and C5, growth of *R. necatrix* was inexplicably greater at 20ppm than at 2ppm, but only C1 and C4 were statistically different. Only 10 of the 17 products reduced the growth rate of *A. mali* by 50% at 200ppm. Again, three of the fractions produced growth rates higher at 20ppm than at 2ppm; these included A1, A4 and C1, but only A1 was significantly different. It is possible that these extracts contained nutrients that the organism could utilise in the presence of lower concentrations of other, fungitoxic cypress compounds.

In the first experiment the length of time for *R. necatrix* to reach the edge of the plate was three days, all subsequent experiments were completed in four days, therefore day three data was used to compare growth rates of all products. There was some variation in the growth rate on the control or non-amended treatment across each experiment. *R. necatrix* may not respond well to being continually subcultured, however, due to the short time frame between the experiments it was not considered appropriate to store and revive the culture. Regardless, the trend was the same in each experiment with no significant reduction in growth until 200ppm.

Recommendation

- No further testing be conducted due to the high concentration required to reduce radial growth of these organisms